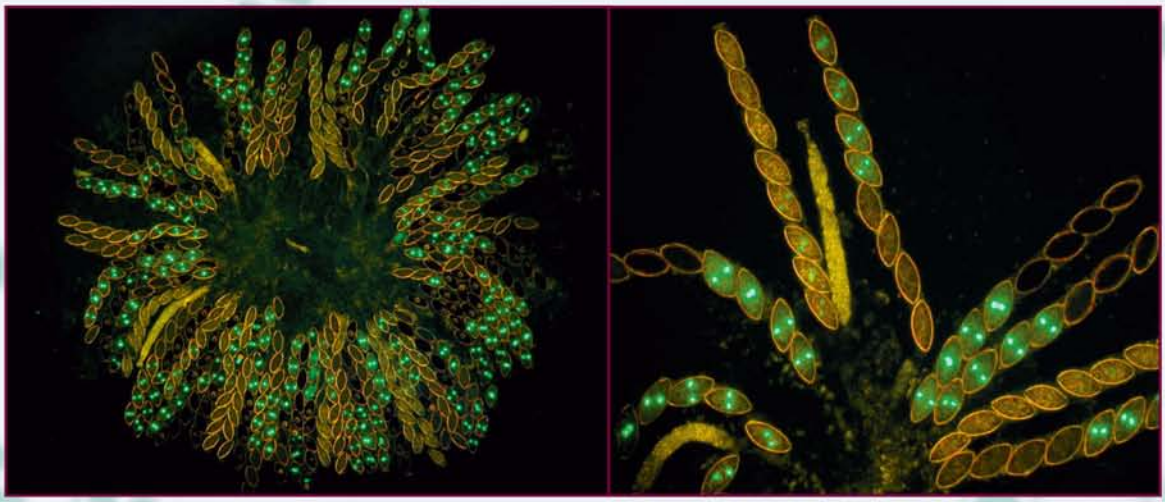
MYCOLOGY VOLUME 24

Fungi

Experimental Methods in Biology



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Fungi
Experimental Methods in Biology

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Cover Image Left: A rosette of asci of *Neurospora crassa* from a cross wild type x *hH1-GFP.* Each ascus contains eight meiotically produced, linearly arranged ascospores. The *Neurospora* chromosomal protein gene histone H1 has been tagged with a gene from a jellyfish, which encodes a green fluorescent protein that glows bright green under blue light excitation, making it possible to visualize nuclei using a fluorescence microscope. *Right:* An enlarged view of asci. Photo courtesy of Dr. Namboori B. Raju, Stanford University.

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Dedicated
to Manjuli and Govind

Preface

Fungi are organisms generally composed of tubes that are invisible to the naked eye. The cells of these tubes are multinucleate and in cytoplasmic continuity. Fungi are among the oldest and largest living organisms, rivaling the mass of a California redwood tree or a blue whale. As the chief agents of decomposition of organic matter, fungi contribute to the sustenance of the carbon cycle. As mycorrhizal partners of roots, they provide the primary mechanism for the capture of nutrients used by plants, thereby contributing to the green cover on earth. Some fungi occur as endophytes in plants or as symbiotic partners with algae, allowing the mutualistic partners to tolerate and grow in harsh conditions that they could not do otherwise. As virulent pathogens of plants, fungi are a constant threat in agriculture and forestry. Since antiquity, fungus has been exploited either unwittingly or intentionally for the conversion of grape juice into ethanol in wine. As producers of antibacterial compounds, fungi are sources of life-saving drugs. They are the only eukaryotic organisms that can thrive at temperatures beyond which no plant or animal can live. Though potentially immortal, a few fungi have a limited life span, providing valuable models for investigating the mechanisms in aging and death. Fungi are now at the forefront of research on mechanisms in gene silencing, biological rhythm, mating processes, biogenesis of intracellular organelles, adaptations to hostile habitats, structure of natural populations and speciation. Because of their small genomes, fungi are being used in "systems biology" to understand the connections between genes, proteins, metabolic and signaling pathways.

This book on fungi is an outcome of my association with graduate students in biochemistry. The majority of these students had little or no previous exposure to fungi and a few not even to biology. I found that students became interested in fungi if an attempt was made to demonstrate fungi in natural situations and how they could be used to understand complex biological questions, in particular if the design of the experiments that were done to obtain information were described. Today's accelerated pace of research, aided by new

instruments and techniques combining the approaches of genetics, biochemistry and cell biology, has changed the character of mycology, necessitating a new approach for the organization of the subject matter and learning about the fungi.

This book should be useful both for a beginning research worker and a professional. The subject matter is divided into six parts, comprising 14 chapters. Each chapter is self-contained and written in a style that enables the reader to progress from elementary concepts to current thinking on a topic. Throughout, attention is drawn to unsolved questions. References are given only to selected publications, primarily for details on the design of experiments that were used to obtain information, and for the identification of some of the key players. Finally, an Appendix gives the principles in naming fungi, estimated to comprise more than 1.5 million species, and of their broad classification. Many authors include the slime molds in the fungi. Since they consist of naked cells, move in amoeboid fashion and ingest particulate food, I have excluded them.

Ramesh Maheshwari
September 2004

Acknowledgments

I express my gratitude to those who kindled, guided, or stimulated my interest in fungi. My father, Professor Panchanan Maheshwari FRS (University of Delhi), introduced me to fungi and guided my pre-doctoral research on the life history of a subtropical rust fungus. My Ph.D. research supervisors, Professors Paul J. Allen and Albert C. Hildebrandt (University of Wisconsin), and my postdoctoral mentors, Professors Alfred S. Sussman (University of Michigan) and David D. Perkins (Stanford University), deepened my knowledge of fungi. David Perkins encouraged me to write this book by giving invaluable suggestions and answering questions through electronic mail. Dr. Namboori B. Raju (Stanford University) and Professor Rowland H. Davis (University of California, Irvine) also answered several queries. Among my former graduate students, Dr. Mahalingeshwara K. Bhat (Norwich) regularly sent me photocopies of several publications and Dr. Amitabha Chaudhuri (Connecticut) contributed Chapter 6. K. Pitchaimani, Kamal Dev and Anthony D'Souza helped to finalize the contents of the book from the viewpoint of graduate students. Kamal Dev scanned published figures, made some new figures and gave much computer assistance. I am indebted to Dr. Keyur K. Adhvaryu, Dr. M.K. Bhat, Dr. Gagan D. Gupta, Dr. Gordon E. Holcomb, Dr. David J. Jacobson, Dr. Durgadas P. Kasbekar, Dr. Martha Merrow and Dr. Namboori B. Raju, who read and commented on some chapters. My wife, Manjuli, helped me cope with my recent physical disability and minimized the gestation period by reading all chapters and making corrections to improve the text. My brother-in-law Arvind Gupta and my son Govind also helped in ways that enabled writing to be completed. When difficulties increased, my colleague Sunil K. Podder encouraged me with a quote by Madame Curie: "Life is not easy for any one of us. But what of that? We must have perseverance and above all, confidence in ourselves. We must believe that we are gifted for something … and this thing, at whatever cost must be attained."

I am grateful to colleagues, staff and students in the department of biochemistry in the Indian Institute of Science for their courtesies and to the Department of Science & Technology, Government of India, for a book-writing grant.

Ramesh Maheshwari

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Part I

The Unique Features of Fungi

Chapter 1

The Hyphal Mode of Life

Fungi are composed of microscopic, tube-like structures called *hyphae* (singular hypha) which grow on substrates such as leaf litter, fallen timber and herbivore dung. The hypha has the shape of a cylindrical tube of even diameter with a tapering tip that branches subapically, with each branch having a tip of its own. By iteration of this modular unit comprising of a tip and a subapical branch (Figure 1.1), a radiating system of hypha called the *mycelium* is formed (Carlile, 1995). At places, hyphae become interconnected by short lateral outgrowths, bringing the entire mycelium into a protoplasmic continuity. The mycelium spreads over and penetrates into the substratum, secreting digestive enzymes which decompose the polymeric constituents of the substratum and absorbing the solubilized carbon and nitrogen and the phosphorus, potassium and sulfur compounds for its growth. This mode of fungal growth is inferred from observations of fungi growing in nature and by examination of cultures grown on nutrient medium solidified with agar (Figure 1.2).

1.1 FEATURES OF HYPHAE

1.1.1 Spread and Longevity

In the U.S. state of Michigan, a tree-root-infecting fungus, *Armillaria bulbosa*, which had colonized an extensive area of forests, was discovered (Smith et al., 1992). This fungus forms mushroom-like fruit bodies at the base of the tree trunk that are honey-colored, hence the fungus is commonly called the honey fungus. The question arose whether a wide area in the forest was infected by an individual fungus produced from a single spore but, since soil is an opaque medium, the area of spread of subterranean mycelium had to be estimated indirectly. In places, thousands of individual hyphae of this fungus aggregate and intertwine to form dark, macroscopic structures called *rhizomorphs* that look like shoelaces (http://helios.bto.ed.ac.uk/bto.microbes/armill.htm). The rhizomorphs have a large food base as they come out from the stump of a dead tree and are therefore able to extend through non-supportive terrain and infect roots of healthy trees (Figure 1.3). Formation of a rhizomorph is a fungal strategy for bringing a large "starter" energy for overcoming the physical and chemical resistance of the plant host and colonizing it. Portions of rhizomorphs retrieved from the soil were placed on a nutrient agar medium to allow the mycelium to fan out. To determine if the rhizomorphs sampled from a large area of forest soil all belong to an individual fungus that developed from a single spore, an intercompatibility test was performed by pairing them in all combinations on nutrient medium in petri dishes—the rationale

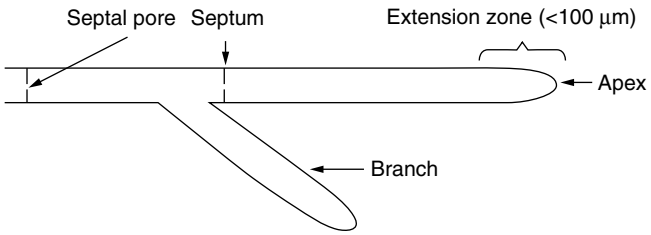

Figure 1.1 Diagram of a hyphal modular unit.

being that if the mycelial growth intermingled to form a continuous mat it would indicate that the isolates were genetically related, i.e., they belonged to an individual fungus. On the other hand, if the isolates showed a zone of aversion, i.e., an area between the paired mycelia not penetrated by hyphae (an incompatible reaction), it would indicate that they were genetically unrelated. Surprisingly, the results indicated that even though the subterranean mycelium of *A. bulbosa* may no longer have a continuous boundary due to the its fragmentation, the mycelial isolates belonged to an individual fungus (Figure 1.4).

1.1.2 Indeterminate Growth

The genetic relatedness of the mycelial isolates was confirmed by DNA fingerprinting method using the techniques of the restriction fragment length polymorphism (RFLP) and

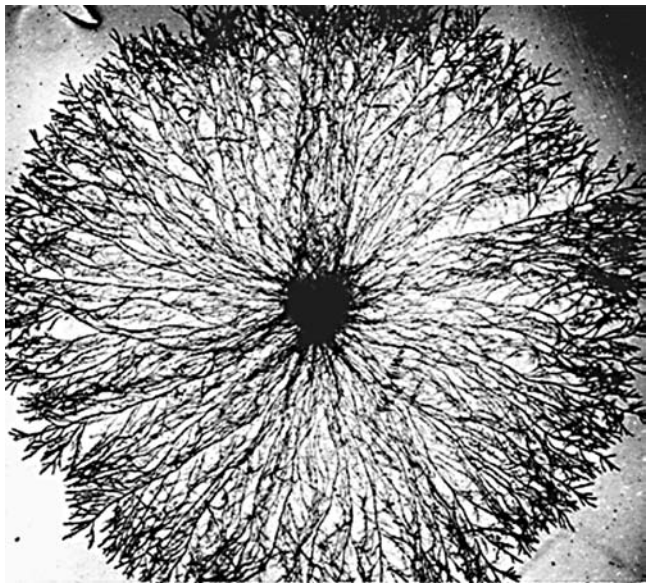

Figure 1.2 A two-day old *Neurospora* colony grown from a small amount of mycelium placed on the center of an agar medium in a petri dish.

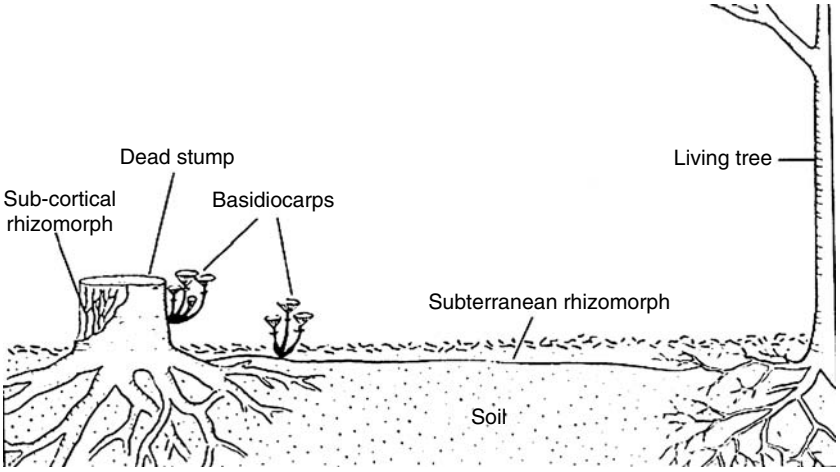

Figure 1.3 Hyphal strategy of attacking forest trees. From Ingold and Hudson, *The Biology of Fungi* (1993), Chapman and Hall, London. With permission of Kluwer Academic Publishers.

random amplification of polymorphic DNA (RAPD). Using RFLP, organisms can be differentiated from one another by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the resultant fragments will differ when the DNA is digested with a restriction enzyme and separated according to their size by electrophoresis. If the pattern of DNA bands differs due to differences in their mobilities, the DNA is from different individuals. In the RAPD method, an arbitrarily designed ten-base-pair sequence is annealed to DNA isolated from the strains and a polymerase chain reaction is carried out. The product is separated on an agarose gel by electrophoresis and the pattern

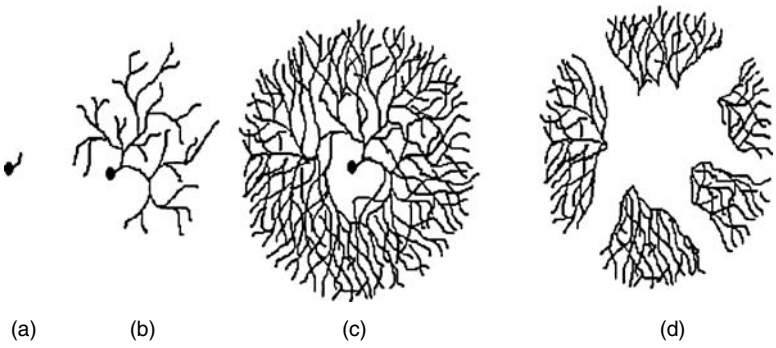

Figure 1.4 Diagram of stages in development of an individual colony of a fungus. (a) A germinating spore (black dot) that has produced a short hypha. (b) Branching to form a radially expanding mycelium. (c) An interconnected hyphal network formed by fusion of hyphae. (d) Disconnected mycelium of an individual fungal colony.

of DNA fragments compared. The finding was that DNA fingerprints of fungal isolates from an extensive area were identical. It was inferred that all isolates sampled from an area approximately 15 hectares belonged to the same individual fungus.

Having ascertained the individuality of this giant fungus colony, the question arose of determining its total mass. From the average weight of the rhizomorph and their numbers in a representative area of the forest soil, it was estimated that this colony of *A. bulbosa* contained at least 10,000 kg of biomass. Furthermore, from the growth rate of the fungus on wooden stakes buried in the soil as well as on nutrient medium, the age of the fungal colony was estimated to be around 1500 years and this large and aged living organism may still be growing. Although less publicized, a clone of *A. ostoyae* discovered in Oregon has a mycelium spread over 890 hectares and is 2400 years old (http://www.anbg.gov.au/fungi/mycelium.html). Since hyphae are capable of potentially unlimited growth, fungi are regarded as immortal organisms.

1.1.3 Apical Extension and Synchronized Growth

> For a fungal hypha, life is at its tip.
>
> *G.W. Gooday and N.A.R. Gow (1990)*

Early in the last century, measurements over a period of the distance between the hyphal tip and the first septum (Figure 1.1) and distances between the successive segments showed that while the former increased the latter remained constant, thereby demonstrating that the growth of hypha is confined to the tip. When ^{14}C-labeled N-acetylglucosamine, a radioactive precursor of chitin—a structural component of the fungal cell wall—was fed to a growing mycelium and its site of incorporation in hyphae was determined by autoradiographic imaging of the whole mycelium, the incorporation of the label was observed only in the terminal region of the hypha. This observation confirmed that a very small terminal region (less than 100 μm) is the growing region of the hypha (Wösten et al., 1991). Cell wall and even organelles in the distal region of the hypha may be broken down by controlled autolysis and the solubilized nutrients translocated to the growing tip for addition into the cell membrane and cell wall at the tip and perpetuation of its growth. The strategy of fungal growth is to keep the tip extending by the active forward movement of the protoplasm. The hallmark of hypha is its growth in one direction only, i.e., its growth is polarized. However, the mechanism by which the protoplasm is drawn toward the tip, leaving an empty tube behind, remains a mystery.

Since the directionality of growth is established at the hyphal tip, this is the likely place where clues to polarized growth of hypha could be found. A variety of approaches are being used: electron microscopy; video-enhanced microscopy of movement of green fluorescent protein (GFP)-tagged organelles in living hypha; fluorescence imaging of distribution of ions; micro-electrode measurement of pH along hypha; patch-clamp detection of ion channels; immunofluorescence detection of enzyme distribution; and measurement of turgor by the plasmolysis method. However, there is no understanding as yet of how the growth of hypha is polarized but some of the pertinent findings are given below.

1.1.4 Spitzenkörper

By phase contrast microscopy and vital staining of living hyphae with a membrane-selective fluorescent dye, a cluster of small vesicles with no clear boundary was observed just beneath the plasma membrane of the hyphal tip (Figure 1.5). This apical body is called

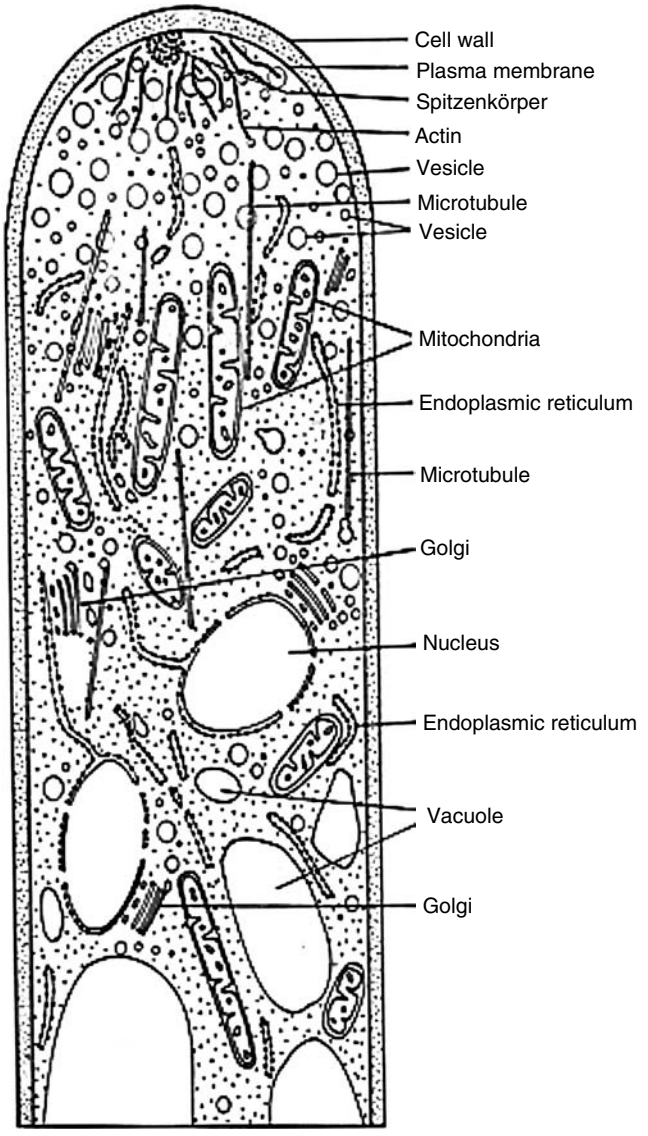

Figure 1.5 Diagram of a longitudinal section of hypha. Adapted from Ingold and Hudson (1993).

Spitzenkörper in German, and advances continuously as the hypha elongates (Figure 1.6). Video microscopy and image analysis of living hyphae showed a close correlation of Spitzenkörper trajectory and the direction of growth of the hypha (Riquelme et al., 1998). Electron microscopy of hyphal tips showed exocytosis of Golgi-derived vesicles to growing tips. From its position and behavior, the Spitzenkörper is considered to be a collection center of vesicles containing enzymes and preformed polysaccharide precursors for cell

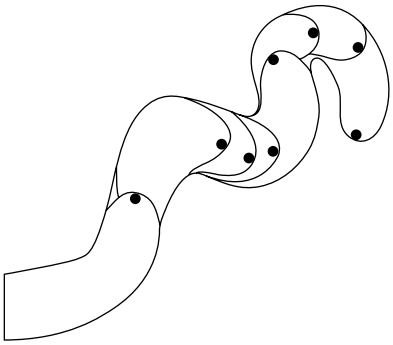

Figure 1.6 Video-imaging of Spitzenkörper trajectory in hypha. Tracings from photographs of *Trichoderma viride* taken during 9 min interval (Bartnicki-Garcia, 2002).

wall synthesis, allowing their localized delivery and polarized growth of the hypha (Bartnicki-Garcia, 2002). Once the Spitzenkörper has discharged its contents, a new Spitzenkörper is reformed. The cycle of collection and discharge of vesicles is consistent with the observation that growth of fungal hypha occurs in pulses (Lopez-Franco et al., 1994). The association of the Spitzenkörper with a meshwork of microtubules and microfilaments suggests that its polarized trajectory is determined by the growing scaffolding of microtubules. Supporting this is the observation that benomyl, an inhibitor of microtubule assembly, markedly disturbed the directionality of hyphal growth of wild-type *Neurospora crassa* whereas a benomyl-resistant mutant was not affected.

The evidence for collection and discharge of vesicles containing membrane and cell wall precursors by the Spitzenkörper is circumstantial. However, their polarized delivery and insertion into the plasma membrane at the tip could explain the generation of hyphal shape and polarity of the hypha. The vesicle membrane and plasma membrane at the tip may have specific proteins which tether the communicating membranes very close for docking and driving their fusion as postulated in animal cells (Rothman, 1994). At the core of pairing between the fusions of vesicle with its target membranes lies an interaction between homologous vesicle and target membrane proteins called v- and t-SNARES. Using specific-antibodies, a tip-high gradient of t-SNARES in *Neurospora* hypha has been demonstrated (Gupta and Heath, 2002). The location of Spitzenkörper at the tip and of tip-high gradient of SNAREs may together bolster rapid apical growth of the tip.

1.1.5 Tip-High Calcium

As in the other tip-extending cells—for example, the plant root hair, pollen tube, rhizoid cell of the alga *Fucus*—the fungal hypha also contains a tip-high gradient of calcium ions (Levina et al., 1995). In hyphae of *Saprolegnia ferax* (an aquatic mold) and *Neurospora crassa* (a terrestrial mold), the cytosolic calcium was measured by ratio imaging emission intensities of the Ca^{2+}-sensitive fluorescent dyes fluo-3 and fura red by confocal microscopy (Hyde and Heath, 1997; Levina et al., 1995; Silverman-Gavrila and Lew, 2000, 2001). Fluorescence emission was localized in the 10-μm region surrounding the tip of growing hyphae but not in the non-growing hyphae. Hyphal elongation was inhibited by microinjection of Ca^{2+}

chelators, suggesting that the tip-high gradient of free calcium is required for tip growth due to an unknown mechanism.

1.1.6 Large Surface Area

Hyphae are generally 5 to 10 μm in diameter. This implies that a hypha has a large surface area in relation to the total mass of protoplasm. The large surface area of hypha maximizes its contact with environment for uptake of raw materials for biosynthesis, for gas exchange and for the release of by-products of metabolism. A large surface area is advantageous in other ways, too. For example, sugars and metabolizing enzymes are stored in the space between the plasma membrane and the cell wall (intramural space), which the hypha can apparently use as the energy and carbon source during exploration of the surrounding area for nutrients. This strategy is manifest in the high respiratory rate of mycelium in the absence of exogenously supplied respiratory substrate (endogenous respiration)—a feature that undoubtedly contributes to tolerance to adverse conditions for extended periods.

The large surface area of the hypha is not without its disadvantages. Because of the single-cell thickness of the hypha, the environment has a direct effect on it, rendering the thin-walled hypha vulnerable to desiccation. Fungi, therefore, must grow either in aqueous media or in a very humid atmosphere. In adverse conditions, the tip perceives a signal and apparently produces a conidiation-inducing factor that diffuses behind it, inducing formation of double septa along the length of hypha. The cells disarticulate and function as propagules called *arthroconidia* (Figure 1.7). Arthroconidia are the simplest type of spores formed by hypha.

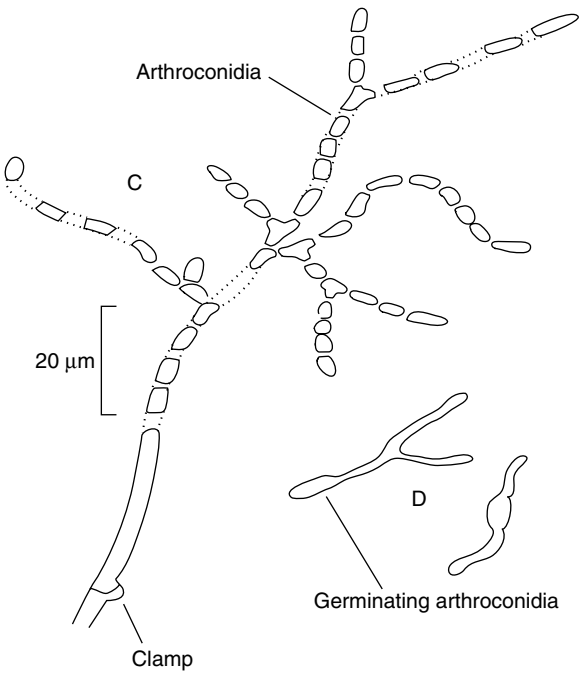

Figure 1.7 Arthroconidia. From Ingold and Hudson, *The Biology of Fungi* (1993), Chapman and Hall, London. With permission of Kluwer Academic Publishers.

1.1.7 Cytoskeleton

Staining of hyphae with anti-tubulin antibody bound to a fluorescent dye, fluorescein isothiocyanate (FITC), showed an extensive array of green-stained, long pipe-like structures called *microtubules*. These microtubules are composed of the protein tubulin and generally lie parallel to the long axis of hyphae (see Figure 1.8). A cytoskeleton element visualized by staining with rhodamine phalloidin is a microfilament (Figure 1.8) composed of the protein actin (Heath et al., 2000). Cytoskeleton in fungi differs from that of animals in that the fungal cytoskeleton is highly dynamic—assembling and disassembling in response to changing cellular needs. These cytoskeletons have a primary role in the migration and positioning of organelles (Riquelme et al., 1998).

1.1.8 Protein Secretion

The substratum on which the hyphae grow generally contains polymeric compounds such as cellulose, hemicellulose, starch and lignin. The enzymes required for depolymerizing

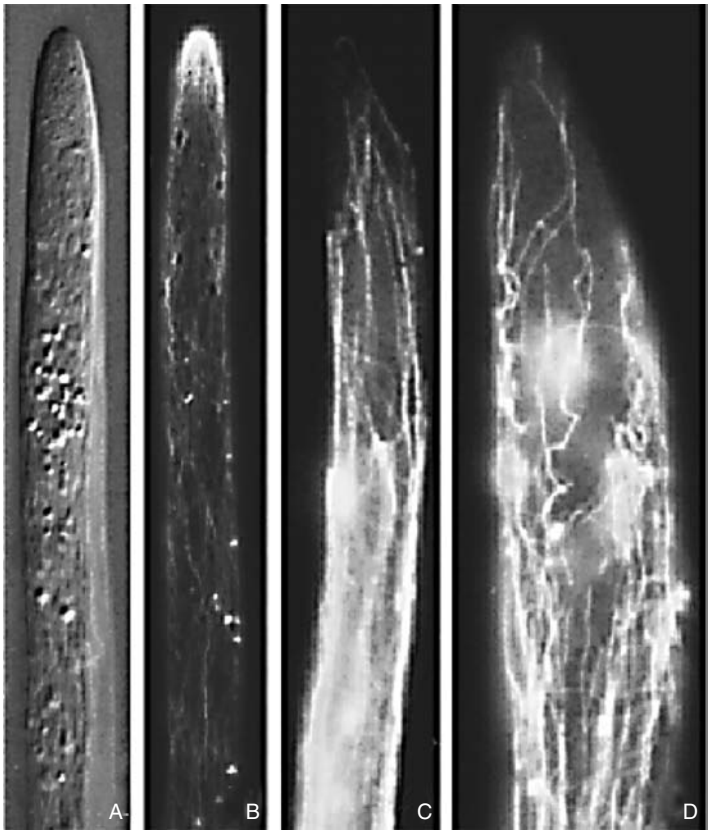

Figure 1.8 Hypha of *Saprolegnia ferax*. (A) Differential interference contrast image of tip. (B) Stained with rhodamine phalloidin showing actin concentrated at the tip. (C) and (D) Stained with anti-tubulin to show sub-apical microtubules. Few microtubules extend to the tip. Reprinted from Heath et al. (2000). With permission of Elsevier.

these polymers are secreted from the hyphal tips. This was demonstrated by growing a colony of *Aspergillus niger* sandwiched between two perforated polycarbonate membranes placed on a starch medium (Wösten et al., 1991). The sandwiched fungal colony could be lifted and exposed to labeled compounds, N-acetyl [^{14}C] glucosamine or [^{35}S] sulfate, washed and used for imaging by autoradiography (Figure 1.9) for monitoring, respectively, the site of chitin and new protein synthesis. This technique allowed simultaneous visualization of hyphal growth and the site of secretion of glucoamylase by immunogold labeling as well as monitoring the zone of starch-degrading activity by I_2-KI staining. The results showed that cell wall synthesis is limited to the growing edge of hypha whereas protein synthesis occurred throughout the hypha. However, Western blotting showed that secretion of glucoamylase occurred only from the hyphal apices. This suggests that the apical region is porous compared to the rest of the hypha and that breakdown of the polymeric compounds is closely connected with apical growth. The large surface area of hyphae and their active protein secretion are features that are being exploited for production of various

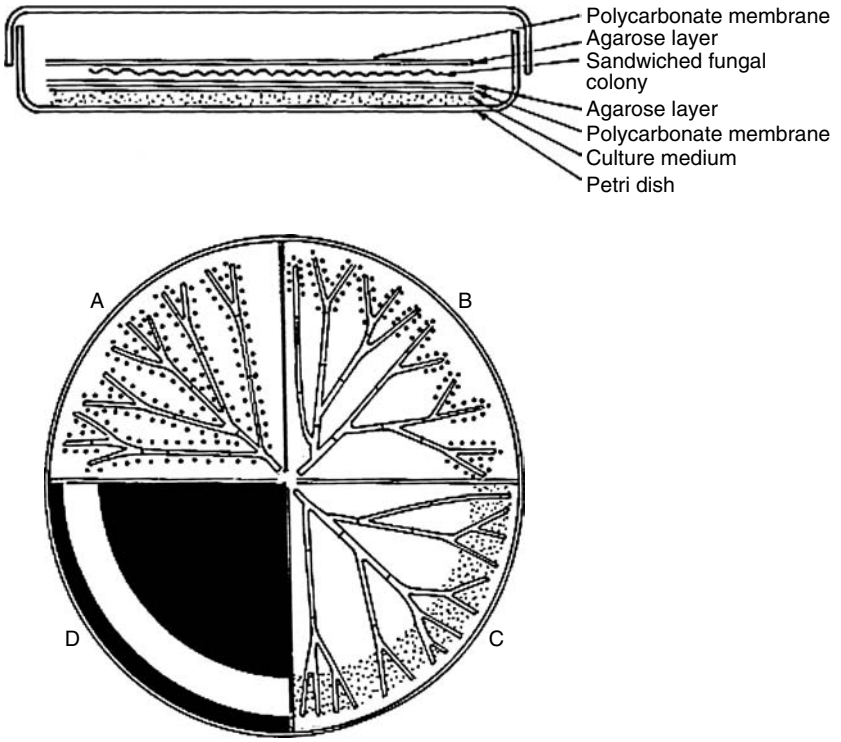

Figure 1.9 Protein secretion at growing hyphal apices. Above, diagram of method of culturing *Aspergillus niger* as sandwiched colony between two perforated polycarbonate membranes placed on starch medium. (A) Diagram of autoradiograph imaging of mycelium for monitoring protein synthesis. (B) Diagram of autoradiograph showing chitin (cell wall) synthesis. (C) Diagram of immunogold labeling showing site of glucoamylase secretion. (D) Diagram of zone of starch degradation by I_2-KI staining. Based on Wösten et al. (1991).

enzymes on an industrial scale such as glucoamylase, protease and xylanase for use in the preparation of glucose syrups, the manufacture of cheese and in the paper industries, respectively. It is envisaged that hyphae of *Aspergillus* sp. will be utilized for production of mammalian antibodies (Ward, 2000).

1.1.9 Nutrient Uptake

In 1974, a miniaturized microelectrode with a very high spatial resolution was used to measure voltage along germinating zygotes of brown algae, growing pollen tubes and root hair cells. It was found that these cells generate a current—a longitudinal pH gradient—in the surrounding medium due to an extracellular flow of a proton (positive charge) that enters the tip and exits from behind (Jaffe and Nuccitelli, 1974). This observation was extended to fungi. Measurement of the pH profile along the hypha of *Neurospora* showed that the apical zone (200 to 300 μm) was relatively alkaline and the distal zone relatively acidic (Kropf et al., 1984; Harold, 1999). It was hypothesized that the fungal hypha is electrically polarized. Protons (H^+ ions), produced because of oxidative metabolism of glucose, are extruded from the rear region of the hypha, making this region of hypha relatively acidic. The extruded protons re-enter from the apex, making it relatively alkaline. The hypha thus drives a current of protons through itself with an inward flow of protons from the tip and their efflux from the distal region (Figure 1.10).

The hypha secretes a variety of enzymes which breakdown the polymeric constituents of the substratum into simple forms by means of extracellularly secreted enzymes. The entry of protons is coupled to the active co-transport (symport) of ions, sugars and amino acids. The rapid internalization of solubilized nutrients is the basis of the absorptive mode of nutrition of fungi. The spatial separation of H^+-pump and nutrient transporters suggest that a hypha is not only cytologically but also physiologically polarized.

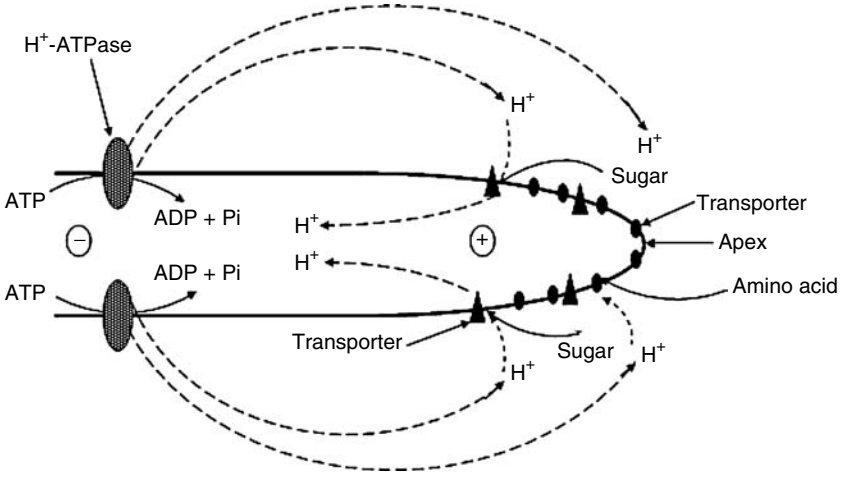

Figure 1.10 Diagram of proton pump and symport in hypha. Based on Harold (1999).

1.2 CELL WALL

1.2.1 Composition and Structure

The tubular form of the hypha is an ideal structure for forcing entry into living tissues, for extending through soil or for growing erect to produce propagules and disseminate them into the air. For carrying out these functions, the hypha must generate enormous turgor pressure and have strong cell walls to contain it. If the cell wall is digested by microbial cell wall lytic enzymes, round protoplasts are released. This suggests that the tubular shape of the hypha is due to the rigid cell wall. The nature of the cell wall polymers providing rigidity to hypha has been studied by extraction with hot acid or alkali and characterization of the solubilized and insoluble material and by successive digestion with specific polymer-degrading enzymes (chitinase, cellulase, laminarinase) and monitoring the appearance of shadowed preparations of hyphae by electron microscopy (Burnett, 1976). The cell wall is composed of polysaccharides and proteins and the major polysaccharide components are glucan, chitin and chitosan. Some fungi belonging to Straminipila (see Appendix) have cellulose also.

Current findings suggest that the fungal cell wall is a layered structure (Figure 1.11). Most fungi have at least three layers: an outermost amorphous layer that is removed by laminarinase treatment and therefore considered to be principally composed of α-(1→3) and α-(1→6)-linked anhydroglucose units. Polysaccharides occur as interwoven microfibrils embedded in an amorphous matrix. The polymers are apparently cross-linked, consistent with the idea that the hyphal wall can withstand an enormous turgor pressure. The composition and structure of the cell wall may vary with age and environmental conditions.

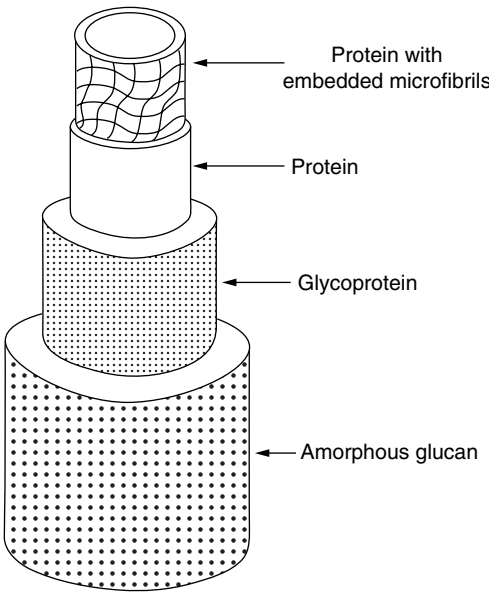

Figure 1.11 Diagram of multilayered fungal cell wall. Adapted from Burnett (1976).

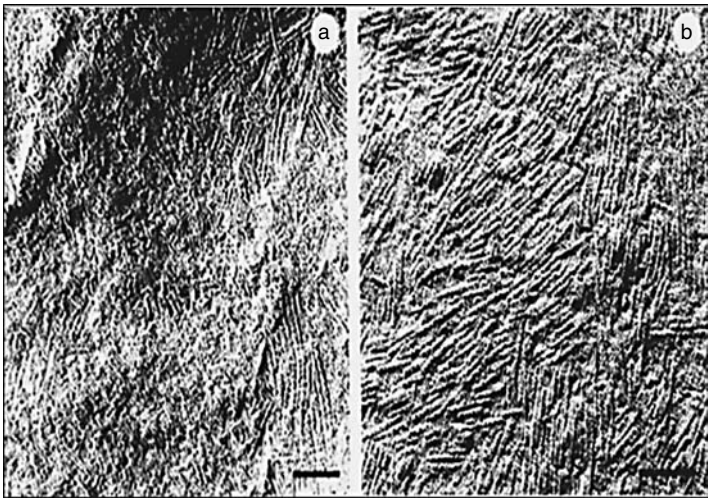

Figure 1.12 Hydrophobin. (a) Electron micrograph of surface of aerial hyphae of *Schizophylum commune* after freeze-fracturing and shadowing showing layer of hydrophobin rodlets. (b) Electron micrograph of rodlets after drying solution of extracted hydrophobin. Source: Wessels (1966). With permission of Elsevier.

1.2.2 Hydrophobins

The hyphae of terrestrial fungi grow in tight contact with substratum to perceive microscopic surface signals and orient the direction of their growth or lower the water surface tension and grow into the air for the formation and dissemination of spores (Talbot, 1997). The hydrophobic molecule that determines such growth of the hyphae is a protein called *hydrophobin*, which is extractable by strong trifluoroacetic acid or formic acid. Hydrophobins have a high proportion of non-polar amino acids that allows them to self-aggregate to form a film that appears as bundles of mosaic rodlets in electron micrographs (Figure 1.12). Hydrophobins were discovered by Joseph Wessels and his associates who estimated that hydrophobins, which are unique to fungi, constitute 10% of total proteins in fungi (Wessels, 1996).

1.3 MYCELIUM FORMATION

1.3.1 Septation

Except in the fungi belonging to Zygomycotina, the hypha is partitioned into cells by transverse walls or *septa* (singular septum). The septa are visualized by phase contrast microscopy or by fluorescence microscopy following staining with Calcofluor white, a cellulose and chitin binding dye (see Figure 2.1). Septa are spaced evenly—for example, in *Aspergillus nidulans* the apical cell (distance from the hyphal tip to the closest septum) is variable whereas the intercalary compartments have a uniform length of 38 μm (Wolkow et al., 1996), suggesting that some cellular mechanisms determine the site of placement of the septa. Septa are formed by centripetal growth of the cell wall and have a perforation (see Figure 1.1) through which cytoplasmic organelles, including nuclei, can pass. In fungi,

the term "compartment" is often used in lieu of cells to denote that fungal cells have cytoplasmic continuity.

The role of septa is uncertain. The fungi belonging to Zygomycotina lack septa. In Ascomycotina and Basidiomycotina, an electron-dense protein-body called Woronin body—a peroxisome-derived dense-core—is present on either side of the septa and functions as a plug for the septal pore in response to cellular damage. By regulated closing or opening of the septal pores, the movement of protoplasm can be redirected to any region in the mycelium, changing the direction of hyphal extension for productive exploration of nutrient-rich surrounding areas. Septa may compartmentalize hypha to reduce leakage when the hypha is ruptured or provide a reducing environment for redox-sensitive enzymes. Septa, as with the rungs of a ladder, may contribute to the rigidity of hypha. For example, the *cross wall less (cwl)* mutants of *Neurospora crassa* are very weak, probably because the contents of a long hyphal cell ooze out as there are no cross walls to prevent "bleeding." In some higher fungi such as the Agarics, the septal pore is of elaborate construction with thickened sidewalls.

Another plausible role of septa is to allow spatial regulation of branch sites and development of reproductive structures by redistribution of nutrients. By the sealing of septal pores, the translocation of nutrients can be redirected toward a developing fruit body. For example, the spores of *Coprinus sterquilinus* that fall on vegetation are taken in by herbivores and voided with dung where they germinate. The hyphae subsequently fuse and form a single three-dimensional interconnected mycelial network that operationally acts as a unit for maximizing extraction of nutrients from the substrate and translocating these to a developing basidiocarp (fruiting body). "Cooperation by fusing mycelia, rather than competition by individual colonies, is the general feature of fungal growth in nature" (Burnett, 1976).

1.3.2 Branching

Branches arise in acropetal succession in proximity, generally sub-apically to the septum. Hyphae tend to avoid their neighbors and grow outwardly from the center. The pattern of branching can be compared to the pattern of branching in a fir tree with a main hypha and a series of branches borne alternately in two dimensions, suggesting a marked apical dominance. As the leading hyphae diverge from one another, apical dominance becomes weaker. Branching allows the hyphae to effectively colonize and exploit its surroundings.

The mechanisms involved in the cellular organization of hypha are beginning to be studied by genetic methods using conditional mutants (see Chapter 2). Mutants with defects in branching are easily recognized because, in contrast to the wild types, they form compact (colonial) colonies. The colonial temperature sensitive (*cot*) mutant was identified as having a temperature-sensitive defect in hyphal growth: it produces colonial (tight, button-shaped) colonies at 32°C but normal wild-type colonies at 25°C. In a nuclear distribution mutant of *Aspergillus nidulans*, branching intensity—which was determined as the hyphal growth unit length (the ratio between the total hyphal length and the number of tips) —was higher in nutrient-rich media than on nutrient-limiting media (Dynesen and Nielsen, 2003). The cytoplasm volume per nucleus is unaffected by substrate availability, suggesting that branching and nuclear division are coordinated to maintain this ratio. An ecological implication of this is that in nature the fungus can explore its surrounding with thin, sparsely branched hyphae (minimum formation of biomass).

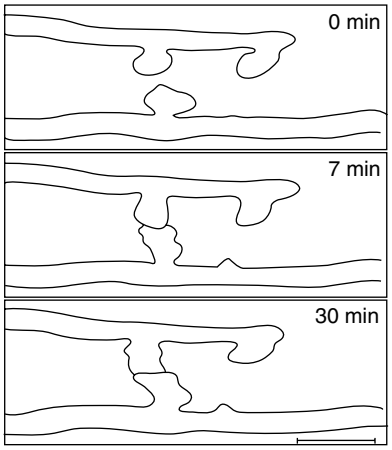

Figure 1.13 Hyphal fusion. Tracings from Hickey (2002).

1.3.3 Hyphal Fusion

As different parts of a mycelium extend, neighboring hyphae may become physically interconnected by cell fusion, bringing all hyphae into a cytoplasmic continuity. The process of hyphal fusion was investigated in living hyphae by time-lapse imaging (Hickey et al., 2002). Short lateral branches arise and redirect their growth toward each other by a remote sensing mechanism to facilitate their contact. This process involves signaling and response of two hyphal tips but the nature of the signaling molecules is not yet known. Since the Spitzenkörper is observed where hyphae meet, this structure presumably delivers enzymes and cell adhesion molecules required for the dissolution and fusion, respectively, of cell wall at the point of contact of hyphal tips to allow for cytoplasmic continuity (Figure 1.13). Formation of interconnected hyphae allows the mycelium to forage in space and in time. Because of hyphal fusion and nuclear intermingling, a mycelium is a three-dimensional mosaic of hyphae of different genotypes (Brasier, 1984).

1.3.4 Multihyphal Structures

In hyphae the cell walls are laid down transversely. Even so, without cell divisions in vertical and anticlinal planes, some fungi form large macroscopic structures (tissue) such as *basidiocarps*, a fruiting body that produces the sexual spores of the mushroom or the bracket fungi[1] (Figure 1.14). These structures are formed by the synchronized growth of thousands of hyphae towards each other with their branching, interweaving, thickening and gluing together by $\beta 1 \rightarrow 6$, $\beta 1 \rightarrow 3$ linked glucan. A feature of fungal morphogenesis is the synchronization of the activities of hyphae but how this occurs is not known.

[1] A basidiocarp of *Rigidiocarpus ulmarius* (over 5½feet, 284 kg in 1996) in a corner of the Royal Botanic Gardens, Kew, Surrey, England is mentioned in the Guinness Book of World Records (http://tolweb.org/tree?group=Fungi&contgroup=Eukaryotes).

Figure 1.14 Basidiocarps of a bracket fungus on the trunk of a mature oak tree. Development of these leathery structures of enormous strength requires the synchronized growth of hyphae toward one another, their localized branching, interweaving, binding and thickening. Courtesy of Heather Angel/Natural Visions. (See color insert following page 140.)

1.4 CONCLUDING REMARKS

> Largely invisible, little studied, the vegetative mycelium of fungi still provides an almost endless series of problems whose investigation is long overdue.
>
> *J.H. Burnett (1976)*

Several questions pertaining to hyphal mode are being actively pursued. How is the hypha constructed as a tube with a tapering apex? What is the function of Spitzenkörper? How is the protoplasm drawn toward the tip? What is the role of microtubules in regulating hyphal extension and direction of vesicles to the polarization sites? How is the site of placement of septum regulated? What is the mechanism of construction of the septal pore? How are the sites of initiation of branches determined? What mechanisms determine that cytokinesis in yeast-like fungi result in complete separation of mother and daughter cells but septation in filamentous fungi leads to generation of individual compartments within the hypha? How does the cell wall extend and what determines the localized secretion of proteins?

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Chapter 2

The Multinuclear Condition

Fungal nuclei measure about 2 to 3 μm in diameter and in the past their small size and poor staining by the conventional cytological stains discouraged their studies. However, recently several DNA-specific fluorescent dyes have been used for routine observations (Figure 2.1). In the last few years, a *Neurospora* chromosomal protein gene, histone H1, was tagged with a gene from a jellyfish that encodes a green fluorescent protein and glows bright green under blue light excitation (488 nm), thus making visualization of fungal nuclei possible under a fluorescence microscope. This method allows nuclei to be viewed in unfixed hyphae and to study the long distance movement of nuclei in living hyphae by video microscopy. The ease of isolating mutants has stimulated studies on the identification of nuclear genes involved in hyphal growth and morphogenesis. The feasibility of fusing fungal cells containing two different nuclear types into heterokaryons makes possible the study of the cooperation or competition among nuclei in multinuclear hypha. Electrophoresis allows the separation of the tiny fungal chromosomes and makes the determination of the chromosome numbers more reliable than was possible by squash preparations.

2.1 NUCLEAR NUMBER AND HYPHAL GROWTH

Unlike plant and animal tissues, nuclear division in fungi is not obligatorily coupled to cell division. As a consequence the hypha, even if produced from a uninucleate spore, becomes multinucleate. Some fungi form spores with varying nuclear numbers. For example, the macroconidia of *Neurospora crassa* contain one to four nuclei per cell. An interesting question is whether the nuclear number influences their rate of germination. Serna and Stadler (1978) measured the DNA content in mithramycin-stained germinating conidia by flow-microfluorimetry as the individual cells passed in a single column through a laser beam. The plot of DNA per 10^6 cells (x-axis) versus time (y-axis) shows a steady rise over a period of several hours, contrary to the expected step-wise increase if nuclear division was synchronous. Most likely the nuclei in conidia are arrested at various points in the nuclear division cycle. The rate of germination of conidia is not related to nuclear number and the significance of the multinuclear condition remains unknown.

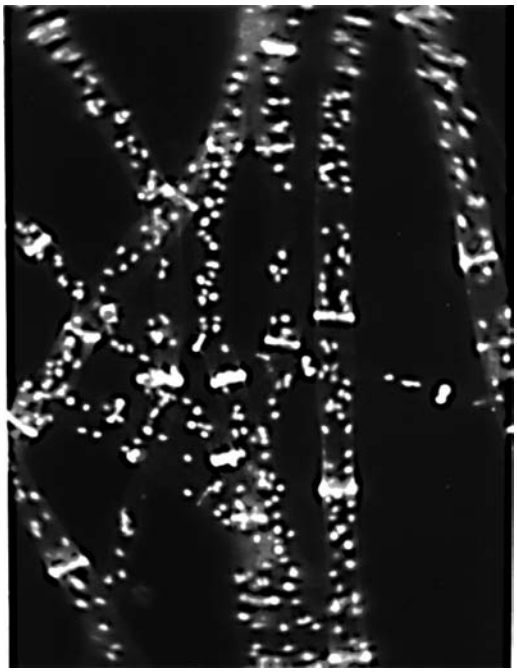

Figure 2.1 Multinuclear mycelium. A portion of *Neurospora crassa* mycelium stained with Hoechst 33258, a DNA-binding fluorescent dye, to visualize nuclei, and Calcofluor, a chitin-binding dye, to visualize septa.

2.2 CHROMOSOME NUMBERS

The majority of fungi are haploid. The genome size of fungi varies from 15 to 45 megabases (Mb or millions of nucleotide pairs of DNA). This genome is smaller than that of other eukaryotes and consequently fungal chromosomes in the vegetative phase are at the limit of resolution of a light microscope. However, in the exceptionally large meiotic cells of certain species belonging to Ascomycotina (e.g., *Neurospora*), the chromosomes are duplicated and condensed. In such cells, the pachytene stage is often used for microscopic determination of chromosome number after staining with aceto-orcein, iron-haematoxylin, Giemsa or acriflavine. Some examples of chromosome numbers (where *n* is one chromosome complement) are: *Aspergillus nidulans* (8), *Alternaria* spp. (9–11), *Cochliobolus heterostrophus* (15), *Magnaporthe grisea* (7), *Nectria haematococca* (*Fusarium solani*) (10–14), *Neurosora crassa* (7) and *Podospora anserina* (7). The unicellular yeast *Saccharomyces cerevisiae* has 16 chromosomes.

The difficulty of resolving intact, tangled chromosomes for microscopic count of chromosome numbers can be overcome by a new procedure (Tsuchiya and Taga, 2001). Protoplasts, generated from germinated conidia using the cell wall-lytic enzyme preparations, are lysed on a microscopic slide to discharge intact chromosomal DNA. The DNA molecules are then separated by *pulsed field gel electrophoresis* (PFGE). In this technique, the orientation of the applied electrical field on agarose gel is periodically changed, thereby reorienting and separating chromosomes. After staining with ethidium bromide, the chromosomes are

resolved as bands and counted under UV light. By this technique chromosome numbers of fungi which lack sexual reproduction—and for this reason the meiotic stage is not available—may be determined. For a PFGE showing separation of chromosomes, see Alexopoulos et al. (1996).

2.3 NUCLEAR DIVISION CYCLE

The cell cycle is divided into four phases: G1 phase (between mitosis and the beginning of DNA synthesis), S phase (the period of DNA synthesis), G2 phase (the interval following the S phase and the beginning of mitosis) and M phase (separation of daughter nuclei). The G1, S and G2 phases are typically a longer part of the cell cycle than the M phase (Figure 2.2). Whereas in plant and animal cells the nuclear membrane breaks down during cell division, fungi—including yeasts—have a closed mitosis: spindle microtubules are inside the nucleus and the spindle pole bodies are formed within an intact nuclear membrane. For a transmission electron micrograph of mitosis in fungi, see Alexopoulos et al. (1996). Since nuclear division in fungi is generally not followed by cytokinesis, some prefer to use the term nuclear division cycle in place of cell cycle for the sequence of events by which the nucleus duplicates. However, because of the common use of the term cell cycle, it will be used here, often interchangeably.

Aspergillus nidulans is an attractive fungus for cell cycle studies because its conidia are uninucleate. Therefore, the rounds of division that a nucleus has undergone at different times can be determined by counting the number of stained nuclei. The first three divisions of nuclei are synchronous and result in the formation of eight nuclei in conidium. In this fungus, three nuclear divisions in conidium are essential for the emergence of a germ tube. The genes that are required for nuclear division as well as nuclear movement into the germ tube are being identified by isolating temperature-sensitive (conditional) mutants in *A. nidulans*.

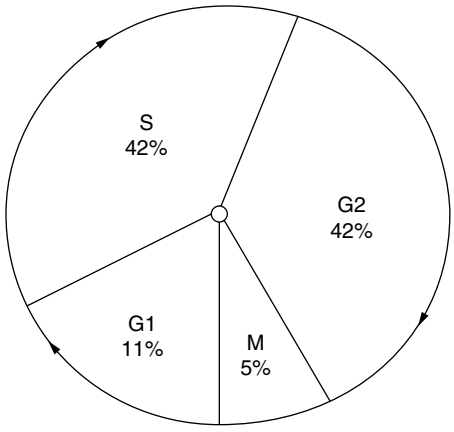

Figure 2.2 *Aspergillus nidulans* cell cycle. The duration of different phases is shown as percentage of total cell cycle.

2.3.1 Temperature-Sensitive Mutants

Nuclear division, nuclear movement and septation are indispensable for hyphal growth. Therefore, to identify the genes that control these events, the mutants in which growth fails only at a higher temperature must be isolated. *A. nidulans* grows normally between 15 to 44°C, whereas a temperature-sensitive mutant does not grow at a higher temperature (44°C) as the mutated gene-encoded protein takes a non-functional or denatured structure. Bergen and Morris (1983) spread mutagenized conidia on agar plates and searched for the rare microscopic colonies in which growth was arrested either before or soon after germination at 44°C but which grew normally if shifted to 32°C (a permissive temperature). When such cells were picked and grown at the permissive temperature, four major classes of mutants were identified: (i) nuclei in the *nim* (never in mitosis) mutants did not divide; (ii) those in the *bim* mutants were blocked in mitosis; (iii) the *nud* mutants were defective in nuclear distribution; and (iv) the *sep* mutants were unable to form a septum following the third nuclear division (Figure 2.3). The mutant approach showed there is no obligatory coupling between nuclear division and cytokinesis. However, nuclear division and septation are required for hypha development as the *nud* and the *sep* mutants fail to grow at 44°C—a temperature at which the wild type can grow.

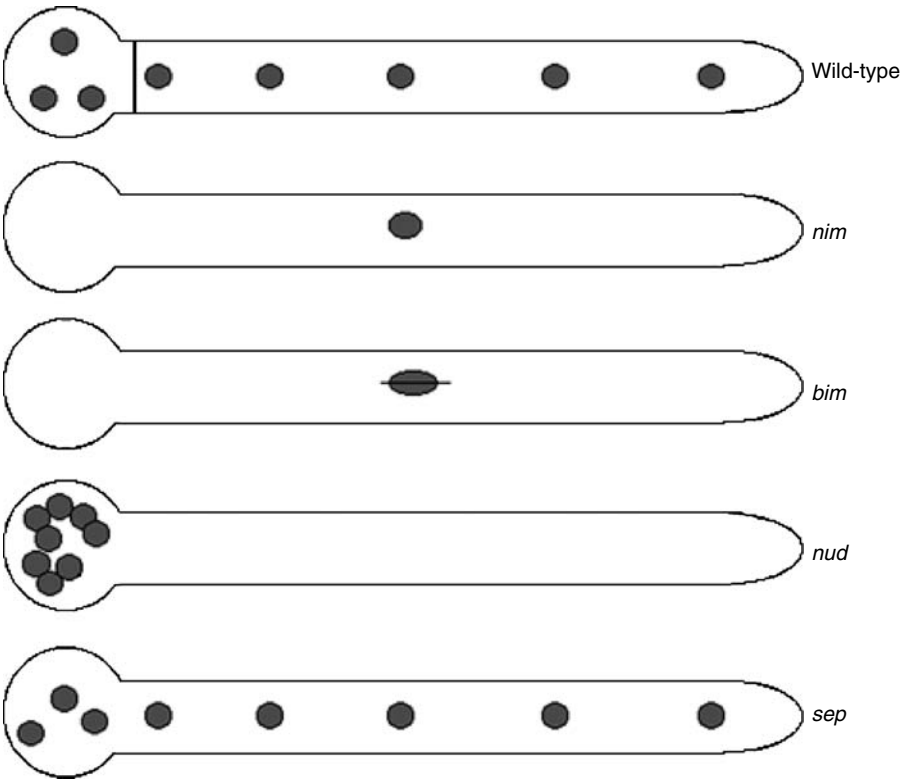

Figure 2.3 Temperature-sensitive mutants of *Aspergillus nidulans* defective in nuclear division and nuclear movement. Redrawn from Plamann (1996).

2.3.2 Kinetics of Nuclear Division Cycle

Using the *nim* mutant of *A. nidulans* blocked in the G2 phase, the nuclear division cycle was deduced from the number of nuclei in the cell (Bergen and Morris, 1983). The first step was to synchronize the multinuclear germlings at the beginning of the S phase. Dormant conidia were germinated for six hours in presence of hydroxyurea (a DNA synthesis inhibitor) at the restrictive temperature. The conidia underwent one nuclear division, indicated by the appearance of binucleate germlings, and this synchronized the germlings at the beginning of the second cycle S phase. The binucleate germlings were shifted to a hydroxyurea-free medium at the permissive temperature and samples removed at intervals and retreated with hydroxyurea before they became sensitive to it, i.e., complete S phase (become tetranucleate). The time required to pass the hydroxyurea-sensitive phase (S phase) was around 40 min. The rate of nuclear doubling was determined from a plot of nuclear division per cell vs. time using the equation:

$$\text{division per cell} = \log\left(\frac{N}{C}\right) \times \log 2$$

where N is the number of nuclei and C is the number of cells analyzed. The nuclear doubling time (generation time or G_t), calculated from the slope of the line was 95 min. The length of M phase, calculated from mitotic index (percentage mitotic figures in hyphal tips) of culture was 5 min. The G2 phase, found by subtracting the aggregate length of M and S from the generation time, was 40 min. The G1 phase, determined as G_t – (S + G2 + M), was 10 min.

In *N. crassa*, morphological differences in the nuclear shape of different phases were used to determine the nuclear division cycle (Martegani et al., 1980). The G1 nuclei are compact and globular; the S and G2 nuclei are ring-shaped and the M phase nuclei are double ring or horseshoe-shaped. Treatment with picolinic acid blocked nuclei in G1. Release from picolinic acid inhibition was followed by a wave of synchronous DNA replication. From the frequencies of phase-specific nuclear shapes, the duration of the G1 phase and S + G2 phase can be estimated. In conidia germinated in sucrose medium, the duplication (generation) time was 100 min (G1 = 20 min, S = 30 min, G2 = 40 min and M = 10 min). The extended G2, relative to G1, is characteristic of fungi. These data on *A. nidulans* and *N. crassa* show that the cell cycle in fungi is completed faster than in animals or plants. For example, a human cell growing in culture has G1= 9 hours, S = 10 hours, G2 = 4.5 hours and M = 30 to 45 min; the complete cycle takes about 24 hours.

2.4 ASYNCHRONOUS NUCLEAR DIVISIONS

Nuclear transplantation experiments with frog eggs and cell fusion experiments with cultured animal cells revealed that nuclear division (mitosis) is induced by diffusible factors present in cytoplasm. For example, when a cell in G1 phase was fused with a cell in M phase, the G1 nucleus in the fused cell prematurely entered into nuclear division (Rao and Johnson, 1970). This observation suggests that the division of fungal nuclei that are in a common cytoplasm is synchronous. Assuming complete synchrony, the uninucleate *A. nidulans* conidia (asexual spores) yields a hypha containing 2^n nuclei, where n is the number of nuclear divisions after germination. A deviation from this value indicates lack of synchrony. Rosenberger and Kessel (1967) found that synchrony was lost after four to six divisions

in individual hypha. In the multinucleate conidia or in the wall-less *slime* mutant of *N. crassa*, synchrony was not observed even when the nuclei were present in close proximity in the same cytoplasm (Raju, 1984). Thus nuclei, although residing in a common cytoplasm, control their division individually.

2.5 NUCLEAR MIGRATION

A process as fundamental as nuclear migration can be expected to have common components in different organisms. In fungi, nuclei must migrate at specific times and in predetermined directions for growth of hypha to occur, for spores to be formed and for mating to take place. Nuclei divide in the hyphal tip (King and Alexander, 1969) and migrate through septal pores into hyphal compartments. The fungal hypha is therefore an excellent material for studying the rates and mechanisms of long distance nuclear movement. Light and electron micrographs show dumbbell-shaped nuclei squeezing through septa or becoming thread-like while entering into a branch. The fungal nuclei may therefore be quite variable in shape. A new technique to study the migration of nuclei is to stain it with DNA-binding fluorescent dyes or tag with the green fluorescent protein (GFP) and monitor movement by video-enhanced fluorescent microscopy. Velocities from 0.1 to 40 μm/min have been observed (Suelmann et al., 1997). Microscopy of live cells often shows nuclei to be stationary in a moving cytoplasm, dispelling the notion that cytoplasmic streaming carries nuclei.

The analysis of fungal mutants provided evidence that nuclear movement requires a motor to move the nucleus through the cytoplasm, a track for the nucleus to move on and a coupling mechanism to link the motor to nucleus (Morris et al., 1995). The *nud* (nuclear distribution) mutants of *A. nidulans* and the *ro* (ropy) mutants of *N. crassa*, respectively, have clustered nuclei rather than evenly distributed as in the wild type. The wild type *nud* and *ro* genes were isolated by transforming mutants at non-permissive temperature with plasmids containing wild DNA and isolating the rare cells that grew into colonies (functional complementation). Plasmid DNA isolated from transformed colonies carried the wild nud^+ or ro^+ gene. Sequencing of nud^+ and ro^+ genes revealed that they encode components of the multi-protein complex called dynein, a mechanochemical enzyme that provides the motive force for movement of nuclei along the filamentous tracks of actin and microfilaments in the cytoplasm (Osmani et al., 1990; Morris et al., 1995; Minke et al., 1999). Given the complexity of molecular motors, several genes are expected to be found that control nuclear migration and their positioning.

The main motor is the large protein dynein, the track is a long cylindrical protein called a microtubule and the coupler is the protein dynactin (Figure 2.4). In one model, cytoplasmic dynein is anchored at hyphal tips and reels in the negative end of microtubules that are associated with the spindle pole body (SPB), which results in movement of the nucleus to the hyphal tip (Plamann, 1996). In a second model, dynein is anchored to the nucleus-associated SPB and pulls the nucleus along a cytoplasmic microtubule track toward the tip. In the third model, dynein anchors to a cytoplasmic microtubule track that is associated with the hyphal tip and translocates a microtubule track that is associated with the SPB. This results in the sliding of the SPB-associated microtubule and movement of the nucleus toward the hyphal tip. Presumably, different motor proteins move nuclei at different velocities. The use of benomyl, a drug that causes disintegration of microtubules, revealed that these proteins are important in nuclear migration. Nuclei move in opposite

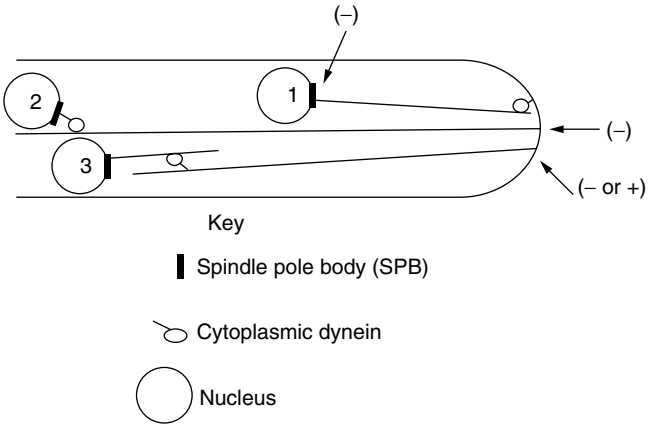

Figure 2.4 Possible mechanisms of nuclear migration. Nucleus #1 is migrating toward the hyphal tip. The negative end of the microtubule (MT) is postulated to be at the spindle pole body. Nucleus #2 has dynein attached to the spindle pole body (SPB) and is migrating toward the fungal tip. This model requires that the negative end of the MTs be at the tip. Nucleus #3 is migrating along an astral MT toward the negative end (at the SPB) and is anchored not at the tip but on a MT that is attached to the tip. (From Morris et al. (1995). With permission from Elsevier.)

directions in hyphal compartments to reach a branch initial, suggesting individual regulation of nuclear mobility (Suelmann et al., 1997). The observation suggested that branch initiation is independent of nuclear distribution although germ tube growth does not occur until a nucleus has entered into it.

2.6 POSITIONING OF NUCLEI AND GENE REGULATION

In the Basidiomycotina, the processes of hyphal fusion, nuclear migration and subsequently the selective association of nuclei convert a monokaryotic hypha into a dikaryotic hypha. The dikaryotic hyphae produce the fruit bodies. A small backwardly projecting outgrowth (hook) occurs from the end cell of hypha into which one of the two daughter nuclei passes (Figure 2.5). Septa form and the hook cell then fuses with the penultimate cell, forming clamp connections at the septa and the process is repeated. The simultaneous division of nuclei, formation of hook cell and its fusion with the penultimate cell ensures that each cell of the mycelium contains two genetically distinct nuclei. Recent work suggests that whether nuclei are juxtaposed or separated can be important in gene regulation (Schuurs et al., 1998). The fruiting body of *Schizophyllum commune* (Basidiomycotina) is rich in proteins called hydrophobins (Chapter 1). The type of hydrophobins secreted is determined by immunochemical staining methods. The monokaryotic hyphae secreted hydrophobin SC3 but not hydrophobins SC4 and SC7. Conversely, the type of hydrophobin secreted by the dikaryotic hyphae is determined on the inter-nuclear distance which could be manipulated by growth on a hydrophobic or hydrophilic surface. On a hydrophilic surface, the nuclei were adjacent (1.6 μm) and the hypha secreted SC4 hydrophobin (which coats

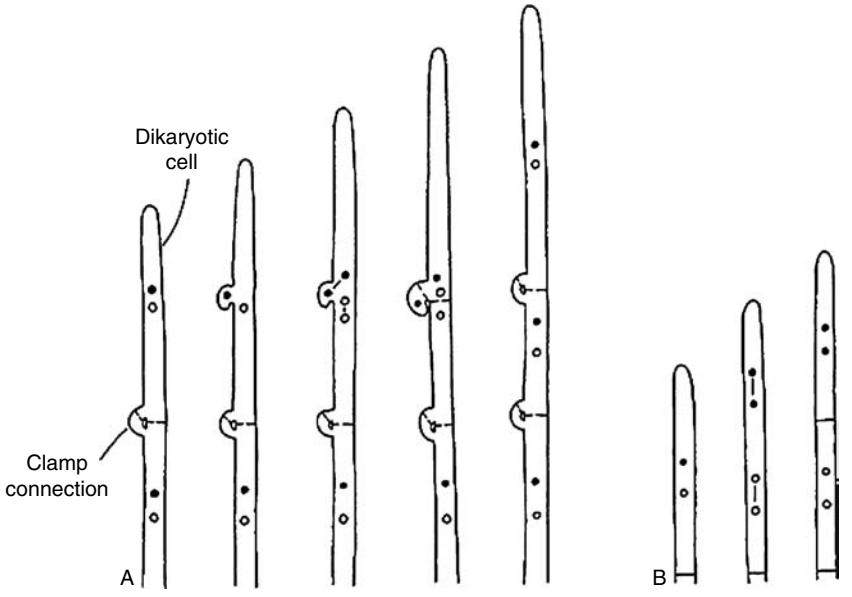

Figure 2.5 (A) Diagram of clamp formation in hypha of a Basidiomycotina. Each cell of the dikaryon has two nuclei of both types. (B) Nuclear types had clamps not formed. (From Ingold and Hudson, *The Biology of Fungi* (1993), Chapman and Hall. With permission of Kluwer Academic Publishers.)

air channels within fruit bodies) and SC7 but not SC3 (which coats aerial hyphae and hyphae at the surface of fruit bodies); whereas on a hydrophobic surface the nuclei were separated (13 to 16 μm) and the hyphae secreted hydrophobin SC3 but not hydrophobins SC4 and SC7. The regulation of gene activity by modulating the internuclear distance could be a unique mechanism in fungal hyphae and could be an important adaptation to changing environmental conditions.

2.7 HETEROKARYOSIS

A consequence of multinuclear condition is heterokaryosis. This term refers to the coexistence of two or more genetically different nuclei inside the same hyphal cell. If single spores of the fungus *Botrytis cinerea* taken from a natural substratum are grown on an agar medium, a circular colony grows which forms sectors, noticeable by poor or heavy sporulation (Figure 2.6). Single spores from each sector produced non-sectoring colonies whereas a spore from the main colony produced a culture that sectored like the parent. Two distinct types of nuclei coexisted in the hyphae and differing types of these nuclei were incorporated in the spores. The heterokaryotic condition can arise either by spontaneous mutation in some nuclei within an originally genetically homogeneous mycelium or by the fusion of genetically distinct hyphae followed by nuclear mixing. Because of their multinuclear condition, heterokaryosis is a unique feature of the fungi. It has been assumed to be an important mechanism in the adaptation of fungi to a fluctuating environment

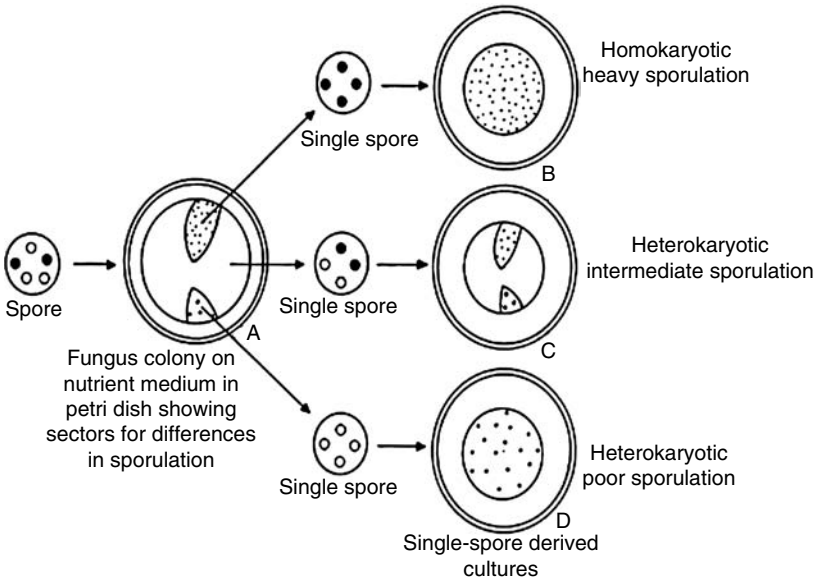

Figure 2.6 Diagram of discovery of heterokaryosis. (A) A colony of *Botrytis cinerea* derived from a single spore producing sectors of poor and heavy spores. A single spore from each sector gave non-sectoring colonies (B) and (D) whereas a spore from the main colony gave a culture sectoring like the parent one (C). This condition is due to different types of nuclei represented by black and white. Redrawn from Ingold and Hudson, *The Biology of Fungi* (1993), Chapman and Hall. With permission of Kluwer Academic Publishers.

through the alteration in nuclear ratios (the proportion of nuclear types). "A fungus which combines heterokaryosis with sexual reproduction provides both for the present and the future" (Burnett, 1976).

In some fungi, co-spotting a mixture of two mutant conidia of same mating type on unsupplemented agar media readily forms heterokaryons. The conidial germ tubes fuse and nuclear mixing occurs allowing heterokaryotic hypha to be formed (Figure 2.7). The formation of heterokaryotic mycelium is used to distinguish if two strains of the same phenotype have a mutation in the same gene or in two different genes in a biochemical pathway (test of allelism). If nuclei with nonallelic mutations coexist in a heterokaryon, the phenotype of heterokaryon is normal since what is lacking in one is present in the other and all functions can be performed. Allelic mutations fail to complement because neither nucleus can perform the vital function (Figure 2.8).

2.7.1 Sheltering of Lethal Mutation

Though the mutation rate is estimated to be on the order of one in one million nuclei, a multinuclear mycelium can over time accumulate lethal mutations. The mutant genes would be sheltered by their normal alleles in other nuclei in the cytoplasm and the lethal mutation can be undetected in multinucleate hypha. Nuclei can, however, be extracted

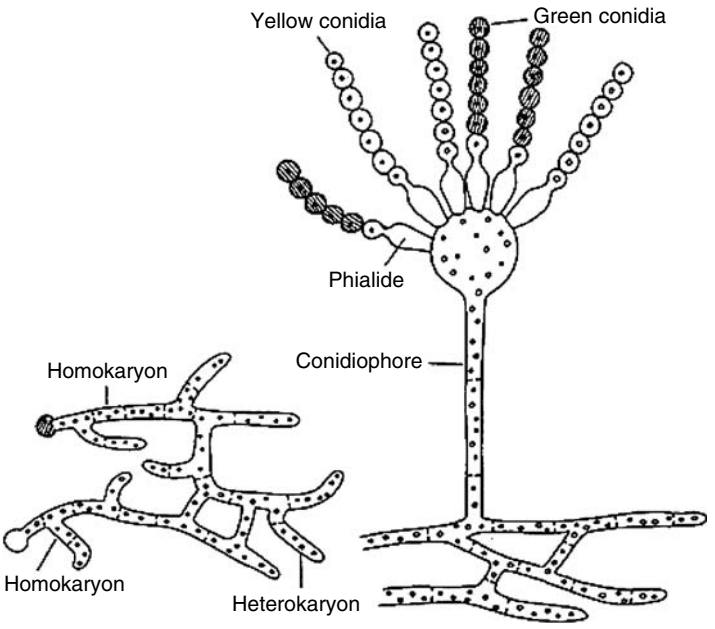

Figure 2.7 Formation of heterokaryon in *Aspergillus*. The two genetically different types of nuclei are shown as open and filled circles. The nuclear types are segregated during the budding of uninucleate spores from phialide cells. The conidia are uninucleate and on germination they produce homokaryotic hyphae that may fuse to give a heterokaryon. (From Ingold and Hudson, *The Biology of Fungi* (1993), Chapman and Hall. With permission of Kluwer Academic Publishers.)

from mycelium in the form of uninucleate spores and these can be grown into a homokaryotic culture and examined for mutant phenotypes (Maheshwari, 2000). An example of this sheltering of a recessive lethal mutation in coenocytic hypha is given in Chapter 14.

2.7.2 Nuclear Selection

Asynchronous nuclear division can result in varying proportions of nuclear types in multinuclear mycelium. Extreme nuclear disproportion can occur in certain genotypes in response to culture conditions. Ryan and Lederberg (1946) constructed a heterokaryon of *N. crassa* containing mutant leucineless (*leu*) nuclei and prototrophic (leu^+) nuclei. When this heterokaryon is grown with leucine supplementation, the mutant nuclei are favored over the wild varieties; the degree of selection is so extreme that the mycelium becomes pure (homokaryotic) leucineless. Either the prototrophic leu^+ nuclei were "inactivated" or eliminated.

Other instances of extreme nuclear disproportion have been discovered. In one experiment, heterokaryons of *N. crassa* having extremely disproportionate ratios of wild and mutant histidine nuclei were used to determine if the enzyme activity encoded by the wild allele is related to the dosage of wild nuclei. A [*his*-3 + *his*-3(*EC*)] heterokaryon was generated by transformation of a histidine auxotrophic strain (*his*-3) with a plasmid-containing

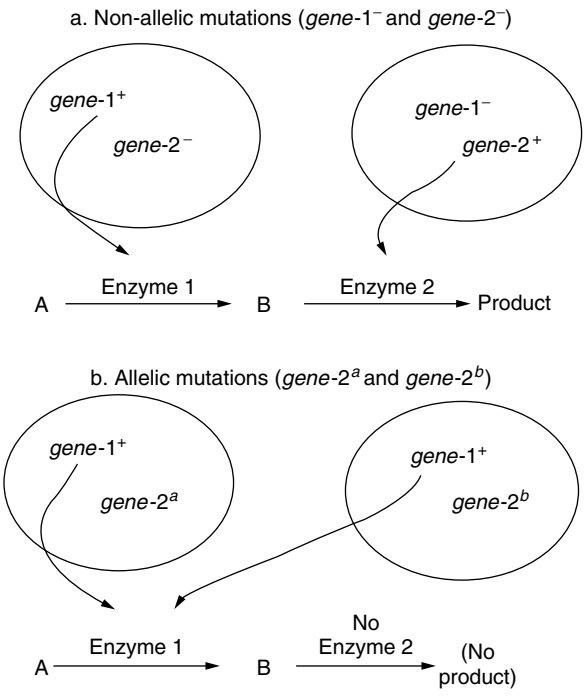

Figure 2.8 The heterokaryon test to determine whether two mutations are in the same gene. (a) Nuclei with non-allelic mutations complement each other, (b) nuclei with allelic mutations fail to complement. (From Davis (2003). With permission of the publisher.)

gene for histidinol dehydrogenase (Pitchaimani and Maheshwari, 2003). (Note that in genetic terminology, the genotype of strains combined in a heterokaryon is enclosed in parenthesis; a *Neurospora* gene that has been integrated ectopically by transformation is designated by appending "EC" to the gene symbol.) Extreme nuclear disproportion occurred in transformant grown in the presence of histidine but not when grown without histidine. Despite the drastic change in nuclear ratio, the activity of the enzyme histidinol dehydrogenase in mycelia grown in the two conditions was similar. The authors hypothesized that not all nuclei in hyphae are active at any given time, the rare active nuclei being sufficient to confer the wild phenotype through biosynthesis of enzyme (Pitchaimani and Maheshwari, 2003).

2.7.3 Nuclear Competence

The reaction of nuclei, even if they differ in a single gene, can be different when combined in a heterokaryon. A phenomenon described as *nuclear competence* illustrates this (Grotelueschen and Metzenberg, 1995). As an example, in *N. crassa* mutant strain 1 is auxotrophic for markers X and Z, while strain 2 is auxotrophic for markers Y and Z. A heterokaryon formed by fusion of strains 1 and 2 is auxotrophic only for the Z marker

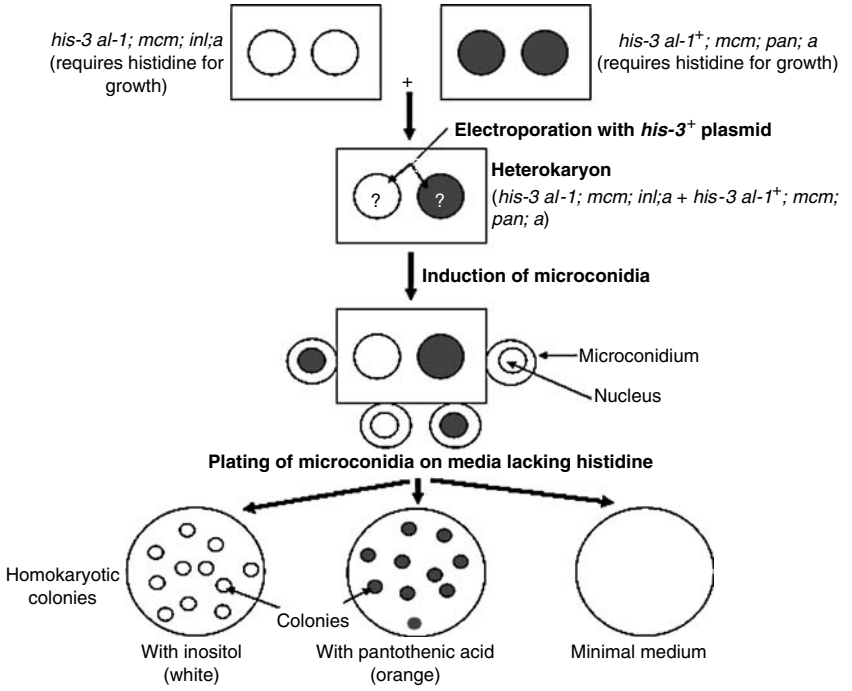

Figure 2.9 Diagram of discovery of nuclear competence. A histidine auxotroph was transformed with *his*-3$^+$ plasmid and resolved into component nuclear types to determine into which nucleus or nuclei the plasmid had entered. Modified from Dev and Maheshwari (2002).

because of complementation of X and Y gene products. When a heterokaryon that contains two nuclear types (Figure 2.9) is transformed with a plasmid carrying an exogenous copy of the Z gene, the heterokaryon became prototrophic for the Z gene product and could be selected on a medium that allows growth only of the heterokaryotic cell. The heterokaryotic cell is resolved into component nuclear types by inducing it to produce uninucleate microconidia (Dev and Maheshwari, 2002). Analysis showed that at any given time, the transforming DNA randomly entered into only one type of nucleus that was "competent" but never into both nuclear types. The results show though in a common cytoplasm, the heterokaryotic nuclei respond differently, most likely because of their different nuclear division cycles where at any given time there is a very narrow window for the entry and integration of exogenously added DNA.

2.8 PARASEXUAL RECOMBINATION

A consequence of heterokaryosis is the parasexual cycle, discovered in *Aspergillus nidulans* by G. Pontecorvo (see Davis, 2003). The parasexual cycle involves the fusion of two genetically unlike nuclei to form a transient diploid nucleus. During subsequent multiplication of the diploid nucleus, a rare event of mitotic crossing-over occurs and members

of each homologous pair of chromosomes assort independently of other pairs. The conidia produced during the cycle are genetically different from the original mycelium as a result of the crossover. The parasexual cycle is not a common phenomenon; however, the general approach of parasexual recombination worked out in *A. nidulans* provided the model for assigning human genes to chromosomes using somatic cell hybrids.

2.9 CONCLUDING REMARKS

A persistent multinuclear condition is a unique feature of fungi. There is as yet no evidence that the multinuclear condition confers an observable advantage of rapid growth rate. Considering that synthesis of a DNA molecule requires chemical energy, the question arises as to why fungi have so many nuclei. Indeed, this question has not received any serious thought or experiment to find an explanation. One possibility could be that the fungi store nitrogen and phosphorus in an organic form (DNA) that is recycled during extension of hypha through nutrient-limited substratum. Among other intriguing questions include: whether all the nuclei are active at any given time; what are the mechanisms involved in migration and positioning of nuclei; how the interaction of genetically dissimilar nuclei that exist in cytoplasmic continuity is affected; what is the influence of the environment in altering nuclear ratios in heterokaryotic mycelium; what is the quantitative effect of nuclei on the phenotype; and what are the mechanisms in the recognition of self and non-self nuclei.

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Part II

Interactions of Fungi with Other Organisms

Chapter 3

Fungi as Symbiotic Partners

> Two organisms don't enter into symbiosis to give something to the partner, but in order to take as much advantage of the partner as possible.
>
> *A. Quispel*

Some fungi live in symbiotic association with cells of higher plants and algae. The mycorrhizal and the lichen-forming fungi are two examples of mutualism. The main physiological basis for this mutualism is bi-directional nutrient transfer. The photosynthetic partner supplies the fungus with sugars and the fungus enhances the ability of the photosynthetic partner to scavenge scarce and immobile nutrients, particularly of phosphorus. Another benefit is the ability of symbiotic partners to tolerate environmental stress and inhabit environments that they could not individually. For example, in Lassen Volcanic and Yellowstone National Parks, where the annual soil temperature fluctuates from 20° to 50°C, plants are colonized by an endophytic fungus *Curvularia* sp. (Redman et al., 2002). Whereas the plants of *Dichanthelium lanuginosum* grown from surface-sterilized seeds in sterile soil that was inoculated with *Curvularia* sp. survived constant soil temperature of 50°C, the non-symbiotic plants died. Re-isolation of the fungus demonstrated that thermal protection is also provided to the fungus.

3.1 MYCORRHIZA

> Land plants never had an independence (from fungi); for if they had, they could never have colonized land.
>
> *K.A. Pirozynski and D.W. Malloch (1975)*

About 90% of all terrestrial plants have underground fungal partners known as *mycorrhiza*, which means "fungus-root" or a root colonized by a symbiotic fungus. The fungus invades tree roots and obtains nourishment by tapping into the plant's vascular system. The hyphae enmesh the root and extend into the soil particles or leaf-litter, tapping a larger volume of soil and increasing the plant's access to water and relatively immobile nutrients. The underground mycelium can even interconnect plants. Many trees are so dependent on their mycorrhizal partners that they languish or die without them. The recognition of mycorrhizal association between roots and fungi provides an explanation for the paradox of the luxuriance of rain forests growing in soil from which soluble minerals have been leached by torrential

rains over millennia and are of extremely low fertility. Mycorrhizal fungi provide the primary mechanism for the uptake of nutrients by the forest trees and thus contribute to the green cover on Earth. In most natural habitats, mycorrhizal plants compared with non-mycorrhizal plants show a greater uptake of mineral ions such as nitrogen, potassium and particularly phosphorus. Mycorrhizal symbiosis is estimated to be several hundred million years old and the primitive root systems of the earliest known plants are associated with fungi. Spores with characteristics similar to that of mycorrhiza are recorded in fossils.

3.1.1 Types of Mycorrhiza

The truffles (Ascomycotina), the agarics and the boletes (Basidiomycotina) are common examples of mycorrhizal fungi. They are now placed in a separate phylum called Glomeromycota or Glomeromycotina. Depending upon whether the bulk of the fungus is outside or inside the root, mycorrhizas are divided into *ectomycorrhiza* and *endomycorrhiza*, respectively. The ectomycorrhiza has a conspicuous pseudoparenchymatous tissue ensheathing the root which sends hyphae between the cortical cells of the root and outwards spreading into the surrounding soil and litter. In a cross section, hyphae are seen penetrating the cortex and forming a dense intercellular network of hyphae, called the *Hartig net* (Figure 3.1). Ectotrophic mycorrhiza are common in the temperate zone. The root tips proliferate and root cells become coral-like in appearance. In endomycorrhiza, the sheath is reduced or absent. Most trees have vesicular-arbuscular mycorrhizal fungi. Arbuscular mycorrhiza are so-called because the fungal hyphae ramify extensively into tree-like structures called *arbuscules* within the root cells, invaginated by the host plasma membrane. The arbuscules provide a large surface symbiotic interface for the exchange of mineral nutrients from the fungus to the plant.

For a hypha to enter into a root, it adheres by the formation of a swollen structure called *appressorium* (Figure 3.2). This appears to be formed as a result of topographical or biochemical signals. Following adhesion, the colonization of the root cortex occurs by an intradical mycelium. The intercellular fungal hypha develops highly branched tree-like fungal structures inside the arbuscules. Arbuscules occupy a major portion of plant cell volume but are separated from the host protoplast by a periarbusular membrane which greatly increases surface area and is the site of nutrient and signal exchange. Arbuscule development in a root cortex cell is accompanied by plastid proliferation, pointing to a highly regulated exchange of compounds and/or signals between the two partners (Hans et al., 2004). Arbuscules degenerate in a few days and the fungus develops ovoid or spherical vesicles which become thick walled and contain fat globules. These asexually formed spores (chlamydospores) are 20 to 1000 μm or more in diameter and persist in the soil for long periods of time. The identification of the vesicular arbuscular mycorrhizal (commonly abbreviated as the VAM or the AM) fungi is based on the size, color, number of wall layers and surface features of the spores. The non-septate, coenocytic mycelium suggests that the VAM fungi belong to the phylum Zygomycotina (see Appendix). The absence of host specificity in VAM fungi suggests that mycorrhizal symbiosis is conserved between evolutionary distant plant species.

3.1.2 Techniques of Studying Mycorrhizal Symbiosis

The study of mycorrhiza is complicated by the biotrophic nature of the fungus. A compartmented pot system has been used to quantitatively estimate the contribution of the mycorrhizal uptake pathway to total plant phosphate (P) supply. Experimental plants (two

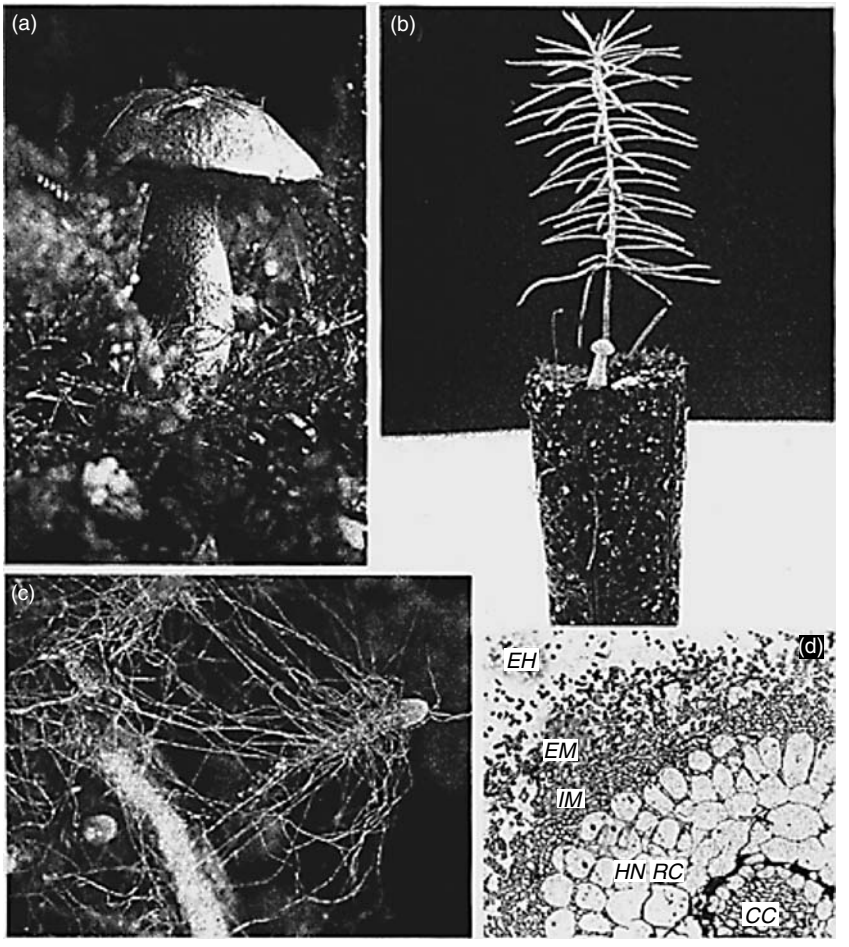

Figure 3.1 Ectomycorrhiza. (a) *Boletus edulis*, one of the Basidiomycotina that forms mycorrhiza. (b) A seedling of Douglas fir (*Pseudotsuga menziesii*) colonized by *Leccinium* sp. The fungal mycelium has developed ectomycorrhizas on the root and has produced a basidiocarp above ground. (c) Short roots ensheathed by an ectomycorrhizal fungus. (d) Transverse section of a Eucalyptus/Pisolithus ectomycorrhiza showing the external (EM) and internal (IM) mantles of hyphae; the fungal hyphae penetrating between the epidermal cells of the root cortex (RC) to form the Hartig net (HN). Extramatrical hyphae (EH) are exploring the medium. From Martin et al. (2001). With permission of Blackwell Publishing.

per pot) were grown (Smith et al., 2003) and a mycorrhizal spore inoculum covered with nylon mesh was placed in the soil, allowing the hyphae but not roots to penetrate into the soil in which ^{33}P-labeled orthophosphate of high specific activity was mixed. The ^{33}P from the soil solution could only reach the plants via the hyphae; unlabeled phosphate could be absorbed directly. After a period of growth, comparison of specific activity of ^{33}P in Glomus-inoculated and uninoculated plants showed that mycorrhizal plants grew better in terms of dry weight production. The pathway of phosphorus transport is thought to

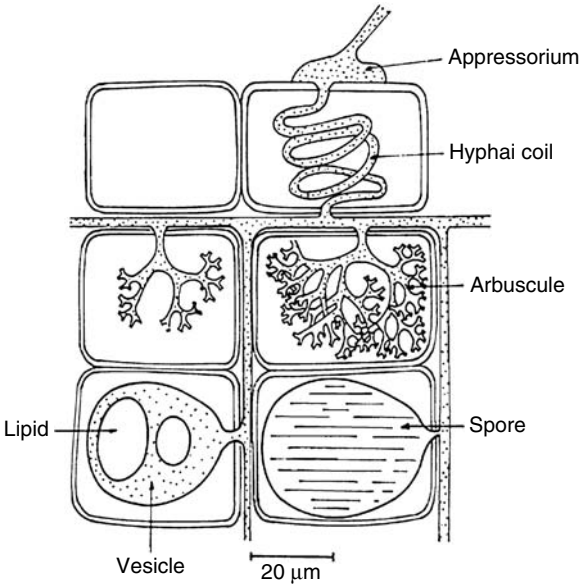

Figure 3.2 Morphology of an arbuscular mycorrhizal fungus. The fungal structures are visualized after staining with trypan blue. Redrawn from Ingold and Hudson, *The Biology of Fungi* (1993), London: Chapman and Hall.

involve the uptake of phosphates by fungal transporters located in external hyphae and then its delivery into cortical cells of the root. Mycorrhizal uptake replaced direct uptake pathways in roots colonized by fungi, presumably due to down-regulation of plant genes encoding phosphate transporters, indicating a molecular cross-talk between plant and fungus.

To illustrate the movement of carbohydrates (Figure 3.3) by experiment, ^{14}C-labelled sucrose was supplied to mycorrhiza via beech root tissue (Harley, 1969). The fungus converted sugars taken up in the root compartment into trehalose—a typical fungal sugar, not found in the higher plants, mannitol and lipids (Pfeffer et al., 1999). From results of such experiments arose the concept of bidirectional transfer of nutrients in mycorrhizal symbiosis: whereas the photosynthetic plant benefits from the transports of phosphates to the root tissue, it supplies the fungus with carbohydrates. The metabolism of the fungal partner was recently examined by combining an *in vitro* compartmentalized system with ^{13}C-labeling and spectroscopic nuclear magnetic resonance (NMR) analyses. Using plugs of carrot roots colonized by *Glomus intraradicus*, isotopically labeled substrates were added either to the roots or the extraradical hyphae which grew out of root and analyzed by NMR spectroscopy. Labeling patterns indicated that ^{13}C-labelled glucose and fructose were taken up by the fungus within the root and converted into trehalose, mannitol and lipids (Pfeffer et al., 1999).

Since the growth of mycorrhizal fungi on artificial media has not been successful, it is assumed they are wholly dependent upon a photosynthetic plant. The *in vitro* root-organ cultures are increasingly used in investigations of VAM symbioses. Several VAM species have been cultivated on root organ cultures (Fortin et al., 2002), providing extraradical

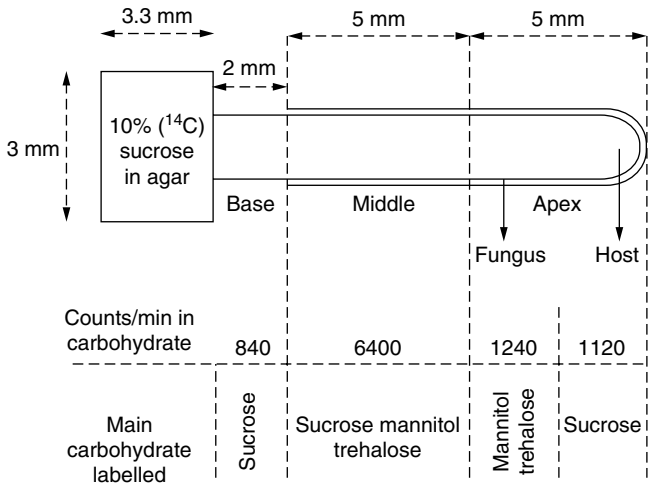

Figure 3.3 Technique to study translocation of ^{14}C-sucrose by root and mycorrhizal tissue. (From Burnett, *Fundamentals of Mycology* (1976), London: Arnold.)

mycelium and spores. One useful technique is to grow mycorrhizal fungi on hairy roots induced by infection with the bacterium *Agrobacterium rhizogenes*. Hairy roots are maintained aseptically in media containing ampicillin. This technique has led to the development of an *in vitro* collection of AM fungi and has been particularly useful for investigating the signaling between the symbiotic partners and the metabolism of the fungus.

3.1.3 Diffusible Host and Fungal Factors

In vitro labeling with $^{13}CO_2$ and NMR spectroscopic analyses shows that dark fixation of carbon dioxide occurs during spore germination (Bago et al., 1996). Therefore, CO_2 emitted by the root tissue is considered as the first non-specific stimulatory compound in the establishment of symbiosis. It is generally held that for further growth and development, the AM fungus is dependent upon the presence of a host (Giovanetti et al., 1993). Certain pea mutants (*Nod*) insensitive to nodulation by the symbiotic nitrogen-fixing bacterium *Rhizobium* are also insensitive to colonization by AM fungi (Albrecht et al., 1999). Using a transgenic plant with a nodulation (Nod)-factor-*gus* reporter gene, it was demonstrated that symbiosis-specific Nod genes are expressed in response to a diffusible factor specific to AM fungi, indicating common controlling genes in the two symbioses (Albrecht et al., 1999; Kosuta et al., 2003).

Attempts to grow spores of AM fungi in nutrient media in the absence of any plant host have been unsuccessful. Using seedlings separated by cellophane membrane and spores of VAM fungi, it was demonstrated that hyphae from germinating spores produce a diffusible factor that is perceived by the roots of the host and stimulate hyphal attachment. Plant exudate containing flavonols stimulate hyphal growth. Though the nature and mechanism of action of these molecules is unknown, it indicates that a molecular dialogue takes place between arbuscular mycorrhizal fungi and their potential host roots. The recurrent theme in symbiosis is exchange benefits for both partners.

The lack of nuclear division correlates with the lack of incorporation of isotopically labeled precursors of DNA and RNA synthesis (Burggraff and Bernger, 1989), although *Gigaspora margarita* is reported to undergo nuclear division when grown *in vitro* even in the absence of a host plant (Becard and Pfeffer, 1993). Root exudates have a strong effect on fungal branching. Flavonols, such as quercetin, kaempferol and myrecetin, strongly stimulate *Gigaspora* species.

The identity of translocated solutes and the factors influencing efflux and uptake are important in understanding the bi-directional transfer of nutrients across symbiotic interfaces of the fungus and the plant. Several years ago, Harley and his colleagues found the ability of the excised beech root mycorrhiza to absorb $H_{32}PO_4^{2-}$ was greater than that of uninfected roots and many times more radioactivity accumulated in the fungal sheath than in the core of root tissue. In short-term experiments, about 90% of the phosphate taken up is retained in the sheath; therefore, the main benefit of symbiosis to mycorrhizal plants is thought to be in phosphorus nutrition. In arbuscular mycorrhizal plants, phosphorus (as orthophosphate) can be absorbed both directly through root and through external fungal hyphae.

3.1.4 Differentially Expressed Plant Genes

Phosphates and hexoses are usually transported by means of symporters. These are sustained by an electrochemical gradient in the plasma membrane created by H^+-ATPase enzymes. Their function is to generate a proton electrochemical gradient across the plasma membrane to provide the driving force for co-transport of phosphate and carbon metabolites through an interface otherwise impermeable to them. Therefore, a key role is proposed for these H^+-ATPases at both the plant and fungal symbiotic interfaces for either phosphate or carbon symport. New forms of transporters are preferentially expressed in arbuscule containing plant cells in response to mycorrhizal infection.

The cloning of a high-affinity phosphate transporter from the mycorrhizal fungus *Glomus versiforme* and its expression in yeast revealed that the fungal phosphate transporter operates by proton-coupled symport at the arbuscular interface (Harrison and van Buuren, 1995). Using a subtractive hybridization technique to identify transcriptionally regulated genes during AM fungal development, a cDNA clone encoding H^+-ATPases from the mycorrhizal fungus *Glomus mosseae* was isolated and its expression compared by Northern blot analysis during appressorium formation, formation of extraradical hyphae and mycorrhizal roots. A developmentally regulated expression was found during mycorrhizal symbiosis. Work is in progress to cytochemically localize the H^+-ATPase in the in planta phase (Requena et al., 2003).

To determine changes in gene expression during symbiosis, the technique of *in situ* mRNA hybridization is used. Murphy et al. (1996) isolated $polyA^+$ mRNA from barley roots in the early stages of colonization by *Glomus intraradices* and used it to construct a cDNA library. The resulting clones were screened with ^{32}P-labelled cDNA, obtained by reverse transcription of RNA from non-colonized and mycorrhizal roots. Colonization resulted in both up- and down-regulation of genes. One of the clones that was up-regulated showed sequence homology with plant H^+-ATPases. This indicates that nutrient uptake by specific carrier proteins via a proton-co-transport (symport) mechanism. Active H^+-ATPases are also present in fungus membranes but how the transfer is polarized across the interface is not known. A possibility is differential activation of H^+-ATPase gene in root cells (Gianinazzi-Pearson et al., 2000).

3.1.5 Multiple Genomes

Staining with 4,6-diamino-2-phenylindole (DAPI) showed that spores of AM fungi, for example, *Gigaspora marginata* and *Scutellospora erythropa*, contain approximately 20,000 nuclei per spore (Burggraff and Beringer, 1989). This number matches well with the amount of DNA extractable from crushed spores and quantifying the DNA on the basis that average DNA content per nucleus is 0.4 picogram (Viera and Glenn, 1990). Despite lack of sex and genetic exchange in the mycorrhizal fungi, surprisingly a single spore contained nuclei with different DNA sequences or several genomes. This was found when one single spore of *Scutellospora castanea* was crushed and the nuclear suspension diluted down to one nucleus per tube (Figure 3.4) for analysis of variation in internal transcribed spacer (ITS) sequences in ribosomal DNA. The ITS sequences are located between the 18S and 5.8S rRNA-coding regions (ITS1) and between 5.8S and 25S rRNA-coding regions (ITS2). Since ITS are flanked by highly conserved coding regions, polymerase chain reaction (PCR) amplification of ITS1 and ITS2 was done using universal primers (Hijri et al., 1999; Hosny et al., 1999). The amplified products were digested with

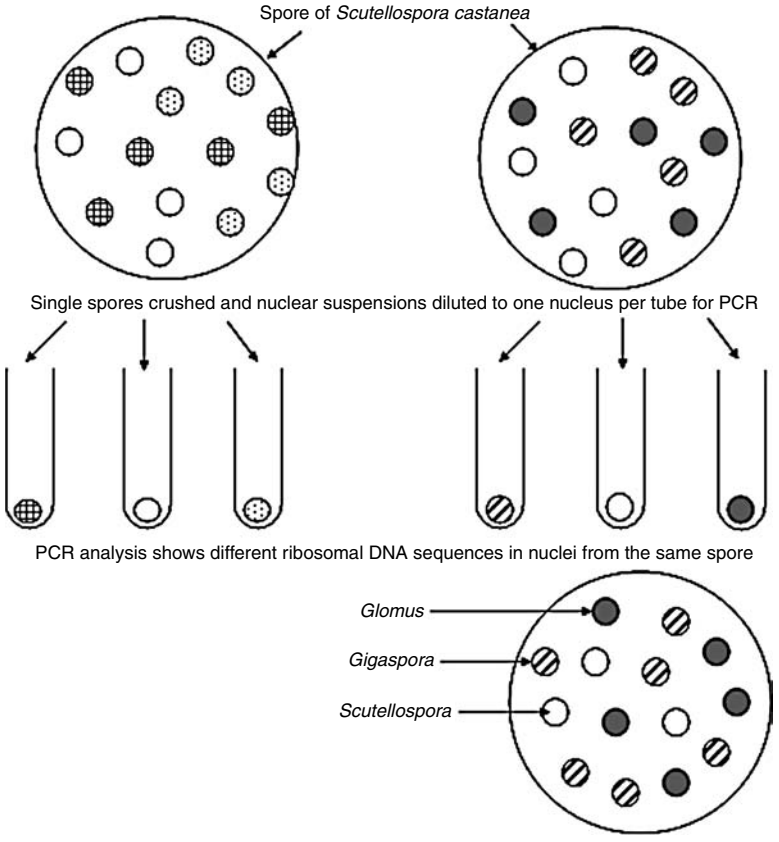
Spore of Scutellospora castanea
Single spores crushed and nuclear suspensions diluted to one nucleus per tube for PCR
PCR analysis shows different ribosomal DNA sequences in nuclei from the same spore
Glomus
Gigaspora
Scutellospora
DNA sequences in S. castanea match genera in Gigasporaceae and Glomaceae

Figure 3.4 Demonstration of a population of discrete nuclei in a mycorrhizal fungus. Adapted from Sanders (1999).

restriction enzymes and fractionated on a gel. Fragments of different lengths (RFLP) showed several ITS sequences among the nuclei, demonstrating that the mycorrhizal spore is heterokaryotic. ITS fragments were grouped into six types that were cloned and sequenced (Hijri et al., 1999). Most of the sequences were very similar to those of *S. castanea*, and a few sequences matched to different *Glomales genera*, *Scutellospora* and *Glomus* (Hosny et al., 1999). This observation raised the puzzling question of how such divergent nuclei have come together in an individual that lacks sexual reproduction. No evidence for sexual reproduction (and hence recombination) has been found, suggesting that they reproduce clonally for their entire association with plants. A plausible explanation is that hyphal anastomoses between mycelia of two genera occurred, followed by exchange of nuclei. Another possibility is mutations leading to creation of different nuclei (Kuhn et al., 2001). Yet another possibility is that the divergent sequences are due to contaminating microorganisms within single spores of arbuscular mycorrhizal fungi (Redecker et al., 1999).

3.2 LICHEN

Lichen (Figure 3.5) is an intimate, symbiotic association of a tangled mass of fungal hyphae (mycobiont) that holds a photosynthetic green or a blue-green algal partner (photobiont) resulting in a stable thallus of specific structure. Crustose lichens are composed of a flat and crust-like thallus. Fungi usually form the basal portion of the lichen, which may be differentiated into a stalk-like structure. The credit of establishing the combination of two dissimilar organisms living together as a single entity is due to the Swiss botanist Schwendener (1872), who said,

> "As the result of my researches, the lichens are not simple plants, not individuals in the ordinary sense of the word; they are, rather, colonies, which consist of hundreds of thousands of individuals, of which, however one alone plays the master, while the rest in perpetual captivity prepare the nutriment for themselves and the master. This master is a fungus of the class *Ascomycetes*, a parasite which is accustomed to live upon other's work. Its slaves are green algae, which it has sought out, or indeed caught hold of, and compelled into its service. It surrounds them, as a spider its prey, with a fibrous net of narrow meshes, which is gradually converted into an impenetrable covering; but while the spider sucks its prey and leaves it dead, the fungus incites the algae found in its net to more rapid activity, even to more vigorous increase …" (see Ahmadjian, 1967).

For nomenclatural purposes, names given to lichens are regarded as applying to their fungal component (mycobiont). *Peltula polyspora* is lichen (mycobiont) and its algal component (photobiont) is *Anacystis montana*, a blue-green alga. The photobiont in lichens is generally a green or yellow-green eukaryotic alga or sometimes a prokaryotic blue-green alga.

Approximately 20,000 species of lichens are known. Lichens grow very slowly—the increase in the radius of the colony of *Xanthoria* is about 2 mm a year. Lichens grow as skins or miniature bushes on rocks and the bark of trees and are colored red, blue, yellow, green and even black with some developing as long grey tufts hanging from branches. The outer layer is formed by fungal mycelium protecting the algal cells beneath from harmful ultra-violet radiation and desiccation. In the center is looser fungal tissue, termed *medulla* (Figure 3.6).

Lichens can survive in the most extreme and severe environments where neither plants nor fungi can exist alone. In the Himalayan mountains, they grow at altitudes of

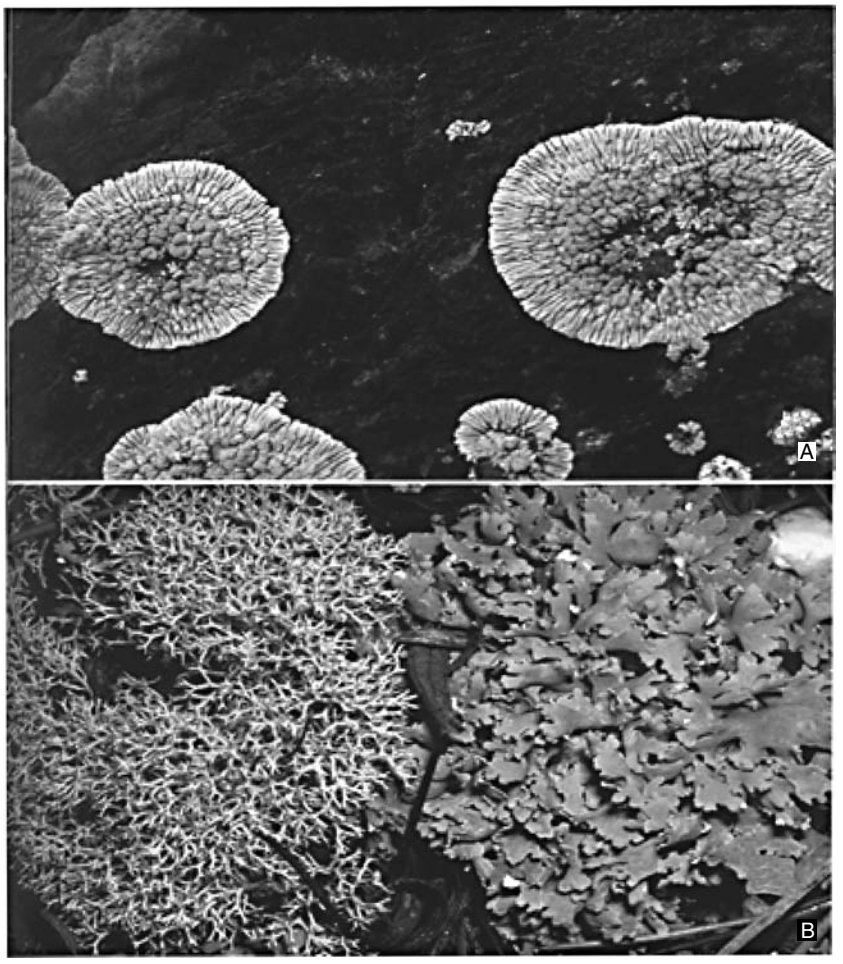

Figure 3.5 Lichens. (A) Crustose lichen growing on rock. Photo: Fred Bruemmer, Montreal, with permission. (B) Lichen about an inch growing as miniature bush or thicket. Photo: Premaphotos Wildlife, with permission. (See color insert following page 140.)

up to 18,000 feet. On the Antarctic ice cap, they have been found on rocks within 300 miles of the South Pole where it is so cold that growth is only possible for a few days in the year. At the other end of the earth in the Arctic tundra, lichens grow with particular luxuriance. A bushy kind, called the "reindeer moss," forms ankle-deep carpets and provides the main food of reindeer in the winter. Lichens obtain their moisture from mists and find all the minerals they need dissolved in the rain. Lichens can also tolerate heat that would desiccate and kill most plants. They shrivel but remain alive and, when the opportunity comes, they take up moisture at extraordinary speed and in great quantities, absorbing as much as half their dried body weight in a mere ten minutes. Most species of lichens are extremely sensitive to sulfur dioxide in the air and are therefore indicators of air pollution.

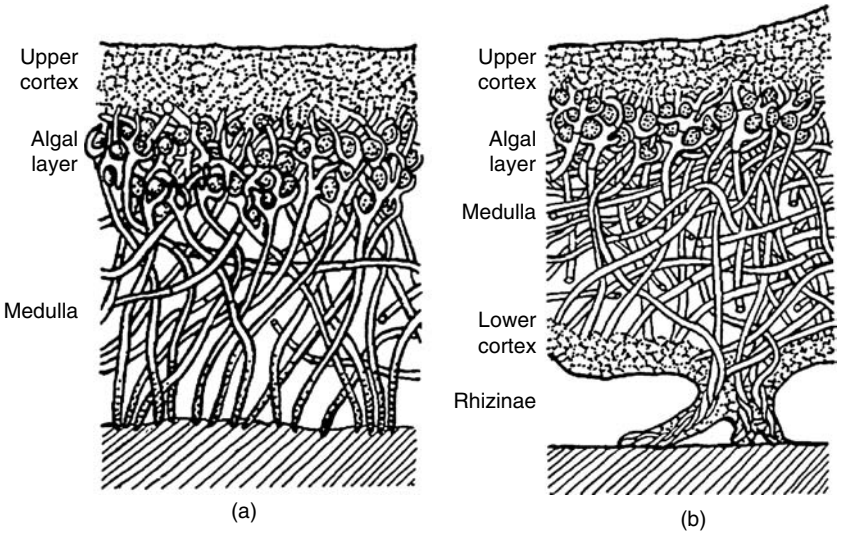

Figure 3.6 Vertical sections of crustose (a) and fruticose (b) lichens. From Ahmadjian (1967). With permission of John Wiley and Sons.

3.2.1 Mycobiont and Photobiont

The mycobiont is mostly an Ascomycotina. The fungus is the dominant member of the partnership and determines the morphology of the lichen. Based on the chlorophyll content of alga, it was estimated that the alga *Nostoc* comprises about 5% of the lichen *Peltigera*. Beneath the algal layer is fungal medulla 400 to 1000 μm thick. Electron microscopy shows that alga and fungus are in intimate contact but there is no penetration of algal cells by fungus. Lichen fungi are not specific to their algal components. Though experimental evidence is lacking, it is believed that mycobionts provide their algal partners with water and minerals and shield the algae from intense light.

3.2.2 Lichen Synthesis

The fruiting bodies of lichens are of fungal origin; therefore, the spores that are discharged give rise only to fungi. A small piece of lichen is fixed to the inner side of a petri dish lid and inverted over the lower half containing an agar layer from which the culture of the mycobiont is established. Lichen fungi grow on a variety of media, although very slowly. There is no marked preference for organic or inorganic sources of nitrogen, disaccharides and polysaccharides.

Many lichen fungi are partially or wholly deficient for the vitamins thiamine and biotin. Some strains on solid media reach a colony size of only 1 to 2 mm in diameter after 9 to 12 months. The temperature range for maximum growth of lichen fungi is 14 to 28°C, the optimum pH is 4.5 to 7.4 and light has no influence. Most isolated mycobionts do not produce spores and are, therefore, difficult to relate to free-living fungi. The synthesis of lichen established the controversy regarding the dual nature of the lichen thallus.

3.2.3 Transfer of Carbohydrate in Lichen Symbiosis

D.C. Smith and co-workers studied the nutrition of lichen thallus by incubating samples of intact lichen under light in solutions of $NaH^{14}CO_3$ and then dissecting the fungal medulla at intervals. Clean preparations of algal symbionts released glucose during photosynthesis. Fixed ^{14}C was found immediately afterwards in fungal medulla, showing that photosynthate moves from alga to the fungus. An "inhibition technique" was developed to identify the mobile carbohydrate between the alga and the fungus (Drew and Smith, 1967). Portions of lichens were permitted to photosynthesize in the presence of $NaH^{14}CO_3$ and a high concentration (1 to 2%) of non-radioactive glucose was added on the basis that it would compete for entry of the photosynthetic product moving from alga into the fungus and this will diffuse out into the medium. Chromatographic analyses of inhibited and non-inhibited lichens showed that [^{14}C]-glucose was detectable after 1 min but not detected after 2 min, being rapidly converted into [^{14}C]-mannitol. Employing this technique with a range of lichens, it was concluded that mannitol, ribitol, erythritol or sorbitol were the mobile forms of carbohydrate; the conversion of fixed carbon into polyol occurred rapidly. Over half of fixed carbon moved from alga to fungus and the main form of carbon stored in the mycobiont is triacyglycerol (Bago et al., 2002).

3.3 SOME UNANSWERED QUESTIONS

The basis of symbiotic partnership are the molecular signals that are exchanged between partners leading to recognition and interactions between phototroph and the fungus. The natures of the molecules involved in cross-talk allowing the partners to live in total harmony with mutual beneficial adjustments are not known. Also not known is how existence in symbiosis causes a massive efflux of particular molecules from the autotroph. The identification of common genes with roles in mycorrhizal and lichen symbioses, the molecular forms and mechanisms by which nitrogen, phosphate and carbon move across the membranes, and the mechanisms by which the fungus forms a carbohydrate sink are among questions that require study. One hypothesis is that the heterotroph directly affects membrane transport systems in the autotroph, generating a strong carbohydrate sink. The possibility of gene transfer between photobiont and mycobiont in lichens needs to be explored. How genetically divergent nuclei arise in arbuscular mycorrhizal fungi in the absence of sexual reproduction is an enigma.

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Chapter 4

Fungi as Plant Pathogens

Plant diseases have been prevalent since recorded history, although the role of fungi as a major causal agent was established only in the nineteenth century. Among the diseases of crops known to the Romans were the rust of wheat and barley, which were ascribed to the sins committed by man, and prayers and sacrifices were therefore offered to appease gods. For example, it was believed that a god named Robigus destroyed grain crops as a punishment for the act of a twelve-year-old boy who had caught a fox robbing his father's hens and punished the animal by tying straw around its tail and igniting it. A ritual was performed in the spring of each year to appease Robigus with the prayer: "Stern Robigo, spare the herbage of cereals; withhold, we pray thy roughening hand," and this was followed by the sacrifice of a yellow dog or any other animal of similar color (Stakman and Harrar, 1957).

4.1 EARLY IDEAS ON PLANT DISEASES

Although wheat rust was sometimes very destructive in Europe, the most generally prevalent disease of wheat was the bunt or the stinking smut (Figure 4.1). In 1750, the Academy of Arts offered a prize for the best dissertation on cause and cure of bunt. It was at that time variously ascribed as due to the use of pigeon droppings, sheep, or horse dung as manure. A French farmer named Matthieu Tillet (1714–1791) put most of these guesses to the test. He divided a piece of land *crosswise* into five equal parts. The first of these he manured with pigeon dropings, the second with sheep manure, the third with night-soil, the fourth with horse manure, and the fifth as control. Next, he divided the land *lengthwise* into four strips. In one he sowed seed deliberately blackened with dust from the bunt, in the second he used seed treated with sea salt and lime, in the third seed treated with lime or with lime and nitre, and the last, which served as control, received seed without any treatment. All the plots that had been sown with seed deliberately contaminated with bunt dust showed a predominance of bunted ears. The plots sown with seed that had been treated with lime, lime and salt, or lime and nitre, were practically free from bunted ears. This solved the riddle; the bunt was no doubt caused by infection of the seed by the black dust from bunt balls. Though Tillet linked the bunt dust with disease and won the prize, he did not realize that a pathogenic fungus causes the wheat bunt. He thought instead that the black dust contained a poisonous principle that could be partially antidoted by saltpeter and lime.

The development of the microscope destroyed the myth associated with plant diseases. In 1807, Benédict Prévost, Academician and Professor of Sciences at Montauban, began

Figure 4.1 Bunt (smut) of wheat caused by *Tilletia*. (A) Healthy and infected spikes of wheat. (B) Enlarged view of diseased and healthy spikes. Smut spores (chlamydospores) formed in masses in spike of wheat. Photo: Department of Plant Pathology, Purdue University.

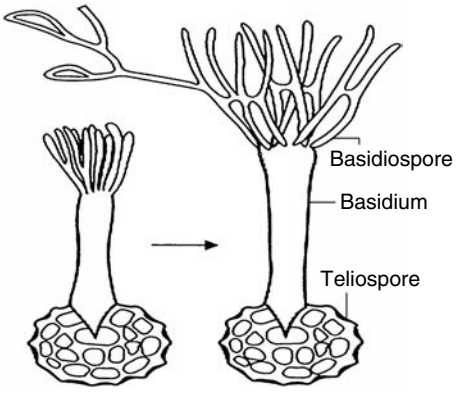


Figure 4.2 Germination of teliospore (chlamydospore) of wheat bunt, *Tilletia*. Haploid sporidia (basidiospores) are at the tip of the germ tube (promycelium). These fuse in pairs via conjugation tube to produce an infective dikaryotic mycelium. (From Burnett, *Fungal Populations and Species* (2003). Oxford University Press. With permission.)

the study of the bunt. He put some particles (spores) from the bunted ears into water and examined them from time to time under the microscope. Within three days, the particles produced a short tube which had a bunch of small shoots at the top that resembled an onion bulb with long narrow leaves, and he called it *hydre végétale* because of its peculiar appearance (Figure 4.2). Prévost noticed that the bunt spores did not germinate in water taken from a copper vessel and thus discovered that traces of copper were toxic to bunt particles. Two brothers, Charles and Louis Tulasne, showed that the particles from the bunt were spores of a fungus. Working in Paris, the brothers studied various kinds of spores and published their microscope observations between 1861 and 1865. They described various kinds of spores produced either simultaneously or in succession by the same fungus.

4.2 SOME STRIKING FUNGAL DISEASES

4.2.1 Diseases of Crop Plants

4.2.1.1 Late Blight of Potato

> We stop the press, with great regret, to announce that potato murrain has unequivocally declared itself in Ireland. The crops around Dublin are suddenly perishing. Where will Ireland be in the event of a universal potato rot.
>
> *Gardner's Chronicle*, September 13, 1845

The potato was once the staple crop of Ireland and people depended on this single crop as their primary food source. In the late summer of 1845, the "fungus" (see Appendix) *Phytophthora infestans* (Straminipila) almost completely devastated the potato crop, resulting in famine. Between 1845 and 1851, almost a million people died of starvation and about a million-and-a-half migrated to America. Because of the economic importance of potato, *Phytophthora infestans* contributed greatly to investigations on fungi as a whole and to the development of the science of plant pathology.

4.2.1.2 Downy Mildew of Grapes

About thirty years after the Irish famine, *Plasmopara viticola* (Straminipila) threatened the vine industry in France. This fungus causes the downy mildew of grapes and was well known in America as a mild pest. In 1876 an American mycologist, W.G. Farlow, warned that if the fungus ever reached Europe, it might prove to be disastrous since the climate in Europe was more favorable to the growth of this fungus. His prediction came true: within two years the first diseased vines were found in France and by 1882 the mold had spread through most of the vine-growing districts. By a rare combination of chance and keen observation, Pierre Millardet, a professor of botany at Bordeaux and a former pupil of de Bary, observed that the conidia did not germinate in water from his well. The reason for the non-germination was traced to a small amount of copper that had leached from the brass pump. He also observed that vine leaves sprinkled with lime and copper sulfate to discourage thievery were free from mildew. Spraying experiments proved that covering leaves and buds of the vines with a mixture of copper sulfate and lime protected the crop whereas unsprayed vines developed infection, showed defoliation and produced no crop. This fungicide since known as the Bordeaux mixture has found wide application.

4.2.1.3 Coffee Rust

Until the middle of the nineteenth century, the consumption of tea and coffee in England was nearly equal. In 1870s, the coffee rust caused by *Hemileia vastatrix* (Basidiomycotina) defoliated leaves and totally ruined the coffee crop in Ceylon (now Sri Lanka). The coffee export to England ceased and the British were forced to change their drinking habit to tea; thus today the British drink more tea than coffee. The coffee rust in Ceylon gave a big boost to tea cultivation by the British settlers in the Indian subcontinent, particularly in the Himalayan mountain ranges and the cultivation of tea in India.

4.2.1.4 Rice Blast

Rice is the staple food for half of the world's population and suffers from a serious threat of the rice blast fungus, *Magnaporthe grisea* (Ascomycotina). Blast disease is threatening Vietnam's winter-spring rice crops nationwide (http://www.cps-scp.ca/riceblast.htm). The fungus is favored by high relative humidity and temperatures around 25°C. It produces brown lesions on leaves, the chief photosynthetic organs in plants, and can be very injurious to plant tissues. The grains are not filled and the entire panicle may die while the blast fungus survives from one season to the next on diseased rice straw and stubble. The entire genome of the fungus has been sequenced at the Whitehead Institute.

4.2.1.5 Corn Leaf Blight

In 1970, the corn leaf blight fungus, *Bipolaris maydis* (*Helminthosporium maydis*) (Fungi Anamorphici) destroyed much of the corn crop in the United States, causing an estimated loss of $1 billion. The disease is favored by moist, warm weather.

4.2.2 Fungal Diseases of Trees

4.2.2.1 Leaf Blight of Rubber

Although the latex-producing rubber tree, *Hevea brasiliensis*, is a native of Brazil, today almost all rubber plantations are in Thailand, Malaya, and Indonesia. Brazil lost its rubber monopoly of the world due to ravages of the dreaded leaf blight, *Dothidella ulei* (Ascomycotina). Attempts to revive rubber cultivation in Brazil were abandoned because of the reappearance of leaf blight.

4.2.2.2 Chestnut Blight

At one time, the majestic American chestnut, *Castanea dentata*, was one of the most important timber trees in the eastern parts of the United States. The wood was easy to work and the nuts served as food. The fungus *Endothia parasitica* (Ascomycotina) was introduced into the United States from Asia and was first observed in 1904 as causing cankers on trees growing in New York City. Within ten years it spread from Maine to North Carolina and westward to Iowa and Nebraska, killing and eliminating chestnut trees throughout (http://www.apsnet.org/online/feature/chestnut/top.html). The fungus is carried from tree to tree as spores and plant quarantine legislation was enacted to reduce the chances of such a catastrophe's happening again. A biological control has promise: hypovirulent strains of the blight fungus were imported from Italy, cultures of which caused a regression when inoculated into cankers. The hypovirulent strains have an infectious viral double strand RNA that is transmitted through vegetative hyphal fusions to lethal strains (Monteiro-Vitorello et al., 1995).

4.2.2.3 Dutch Elm Disease

Another disease of concern has been the Dutch elm disease (first seen in the Netherlands, hence the name) and has spread through continental Europe and into the United States. It is caused by the fungus *Ophiostoma ulmi* (http://helios.bto.ed.ac.uk/bto/microbes/dutch-elm.htm) and is spread by beetles as they carry fungal spores on their bodies from infected trees to the bark of healthy trees where they feed and breed. The fungus spreads rapidly in the xylem vessels as infected trees first show wilting, curling and yellowing of leaves in small branches in the upper portions. Another mode of transmission of the fungus is through the root system via natural root grafts between trees growing close together, where the fungus spreads through water conducting vessels (xylem cells). The tree forms gums within these vessels in response to the presence of the fungus, obstructing water movement and causing the tree to wilt, its leaves to turn yellow and drop off and the eventual death of the tree. Among the fungal diseases of trees, the Dutch elm disease caused by *Ceratocystis ulmi* (Ascomycotina) is an important disease of American and European species of the elm trees. A large number of oak trees prized as shade trees in the midwestern United States have been killed by the oak-wilt fungus, *Ceratocystis fagacearum* (Ascomycotina), which clogs vascular tissues, preventing water movement to the crown and results in the wilting of leaves and finally in the death of the tree.

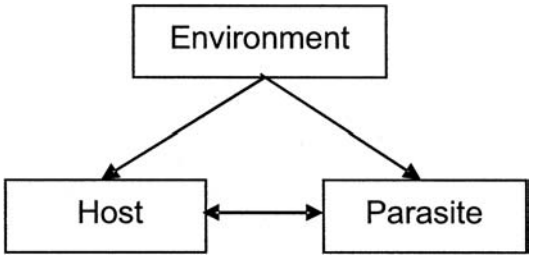
4.3 CLASSES OF PLANT PATHOGENIC FUNGI

4.3.1 Necrotrophic and Biotrophic Fungi

Plant parasitic fungi may be broadly classified as *necrotrophic* or *biotrophic* fungi based on their modes of nutrition. Necrotrophic fungi are destructive parasites and derive their organic nutrients from the dead cells they have killed. Their effects vary from local discrete lesions, as seen in some leaf spot diseases, to massive tissue destruction, as seen in fruit rots. Upon infection, the hyphae produce polygalacturonases and pectate lyase enzymes which act on pectic substances of the middle lamella and release galacturonic acid residues that form the main source of organic carbon used by these fungi for growth. The dissolution of middle lamella leads to partial degradation of the host's cell walls, separation of cells, loss of turgor and their death. Several necrotrophic fungi, such as species of *Polyporus*, *Fomes* and *Ganoderma* (bracket fungi, Basidiomycotina), kill and then live on dead trees as decomposer saprophytes producing ligninases, cellulases and hemicellulases. With the structural cellulose microfibrils in cell walls weakened, the tree topples under high winds. The fungus continues to decompose the trunk as a saprophyte, producing its basidiocarps on the fallen and standing parts of the trunk for a number of years, blurring the distinction between a parasite and a saprophyte. The biotrophic fungal parasites, in contrast to the necrotrophic parasites, can only grow on living hosts. They are termed obligate parasites that include the downy mildews (e.g., *Peronospora*, Straminipila), the powdery mildews (e.g., *Erysiphe*, Ascomycotina) and the rust fungi (e.g., *Puccinia*, Basidiomycotina).

4.4 GENERAL FEATURES OF PATHOGENESIS

Once fungi were recognized as a pathogen, it was understood that disease is a state of altered metabolism of cells and tissues resulting from a reciprocal interaction of a parasite and host, each of which is individually influenced by the environment.



The example of a biotrophic fungus (for example, a rust fungus) can be used to understand what is conveyed by the above diagram and to point to some generic features of pathogenesis. After arriving on the leaf surface, the urediospore germinates if free water is available and the weather is mild. For infection to occur, the germ tube must find a portal of entry. The urediospore germ tube always penetrates through a natural opening in the host. However, contrary to earlier notions that held to the random encounter of germ tube with stomata or a volatile chemical emanating from the tube, the host itself contributes to fungal penetration by directing the growth of the germ tube toward itself. When the germ tube encounters a stomatal pore, a bulbous structure termed the *appressorium* is formed from the tip of the germ tube that is appressed to the plant surface (Figure 4.3). From the underside of the appressorium, a short infection peg is formed which forces entry into the leaf (Maheshwari et al., 1967a, b). The infection peg enlarges into a substomatal vesicle from which infection hypha develop that ramify intercellularly and form specialized structures called haustorium that penetrate the plant cell and through which nutrients from the living cells of the host are absorbed. The morphological transition from a germ tube into infective hyphae that occurs in response to thigmotropic stimulus is crucial for the initiation of parasitic

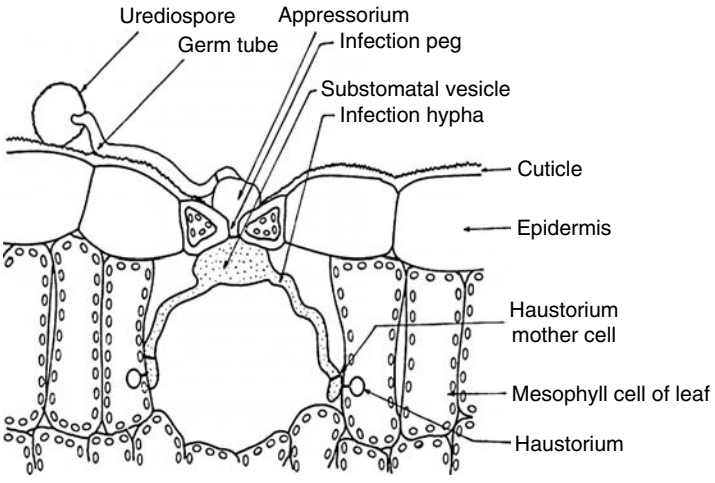


Figure 4.3 Diagram of infection structures of a rust fungus (*Puccinia*) formed on leaf. The leaf is shown in vertical section.

mode of life. Once an interface between the host and fungus is established, signaling mechanisms begin to operate either by initiating defense reactions that limit the colonization of tissue by fungal mycelium or the fungal mycelium overcoming these reactions and acting as a metabolic sink into which photosynthates (sugars and other host metabolites) from uninfected parts are redirected at the cost of their normal translocation pattern toward developing grains, fruits or storage organs. The formation of pustules containing spores is a morphological disruption of host tissue. The respiratory rate of the infected plant tissue is increased while photosynthesis is reduced, resulting in the grains or fruits being shrivelled or not being formed at all. The plant, in common parlance, is diseased.

4.4.1 Adhesion of Spores

Though the prolificacy of spore production might seem to ensure success to a potential pathogen, the pathogen must avoid being dislodged by wind and rain before germination and penetration into a host. Therefore, adhesion of spores and germ tubes is a critical stage in the pre-infection process. The conidia of *Magnaporthe grisea*, causal agent of the rice blast disease, adhere to surfaces as shown by their inability to be flushed off soon after their deposition on a Teflon film that resembles the rice leaf in some properties (Hamer et al., 1988). Electron microscopic examination shows the periplasmic space of the dry conidium contains mucilage at the tip that is rapidly released upon hydration that could allow the attachment of conidia to a hydrophobic surface.

4.4.2 Directional Growth

The germ tubes of some fungi (for example, those of urediospores of the rust fungi) always enter the host plant through stomata. Rather than the chance encounter of stomata, the growing germ tube is directed toward stomata by the surface properties of the host surface. The presence of hydrophobins (Chapter 1) in the hyphal wall allows close contact of the germ tube with the host surface and senses the surface configurations. The cuticle surface is ridged (Figure 4.4) due to oriented deposition of cuticle wax and the germ tube extends perpendicularly to the ridges. The homing of the germ tube on the stomata opening is guided by the orientation of the ridges around the guard cells (Maheshwari and Hildebrandt, 1967a). To test this hypothesis, Hoch et al. (1987) microfabricated 0.5 μm ridges on silicon wafers by electron-beam lithography and placed urediospores on it. The hypha grew perpendicularly to the ridges, implying that hypha could sense minute differences in the leaf surface and orient its growth toward stomata. A sharp change in the elevation around the stomata pore is the topographical signal for the differentiation of the appressorium, a bulbous swelling of the tip of the germ tube (Figure 4.5), over the stomata. It had long been assumed that foliar penetration of germ tubes results from their chance encounter with stomata opening. However, the observations of germinating urediospores suggests that fungi have evolved mechanisms to perceive surface topography and orient the growing hyphal tip towards stomata. One feature of germ tube growth is a close adherence to the surface. Directional growth was also observed on isolated epidermis or on rubber replicas of leaf surface, showing that the germ tube perceives minute features of a host surface to orient their growth toward natural openings in the host (Maheshwari and Hildebrandt, 1967a; Maheshwari et al., 1967b).

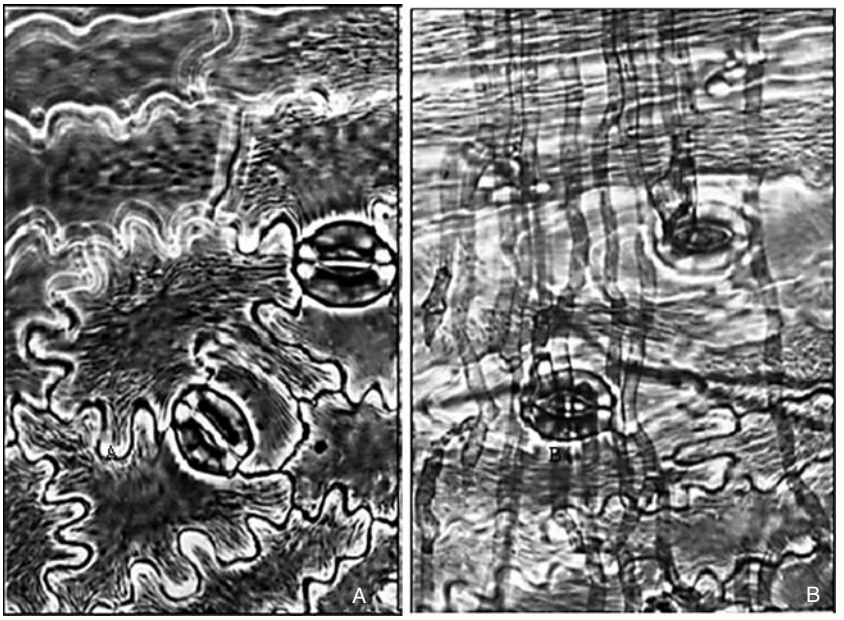


Figure 4.4 Directional growth of germ tubes of rust fungi on a leaf surface. (a) Surface view of leaf epidermis showing cuticular ridges. (b) Urediospore germ tubes oriented perpendicularly to ridges. (From Maheshwari and Hildebrandt (1967).)

4.4.3 Infection Structures

Nearly all fungal plant pathogens gain entry into their host via a structure called the appressorium. The firm attachment of the appressorium to the hydrophobic host surface prevents lift-off as the infection (penetration) peg from the underside forces entry through the narrow stomata pore or pierces through the cuticle and cell wall. Mutants of *Magnaporthe grisea* that are deficient in hydrophobin show reduced pathogenesis (Talbot et al., 1996). Howard et al. (1991) used solutions of polyethylene glycol to plasmolyse and by this technique estimated the force generated in appressorium produced on Mylar membrane (composed of polyethylene terephthalate) was in excess of 8.0 megapascal. The fungus apparently converts the lipid and glycogen reserves into glycerol that causes water uptake and the build-up of turgor pressure. The appressorium cell wall contains melanin, which reduces the porosity of the appressorium cell to solute flux but not water flux and contributes to osmotically generated increase of turgor pressure (Howard and Ferrari, 1989).

The signal for appressorium differentiation induces division of nuclei and a sequence of cellular development that results in the formation of a substomatal vesicle, formation of infection hyphae (Maheshwari et al., 1967c) that develops a projection called the haustorium that penetrates into the host cell, surrounded by an invagination of the host plasma membrane (Figure 4.6). Haustoria are especially prominent in obligate parasites: downy mildew (Straminipila, order Peronosporales), powdery mildew (Ascomycotina, order Erysiphales) and rust fungi (Basidiomycotina, order Uredinales). In powdery mildew, all the hyphae are on the surface of the host and only haustoria penetrate the epidermal cell and are therefore the site of the uptake of nutrients. The formation of

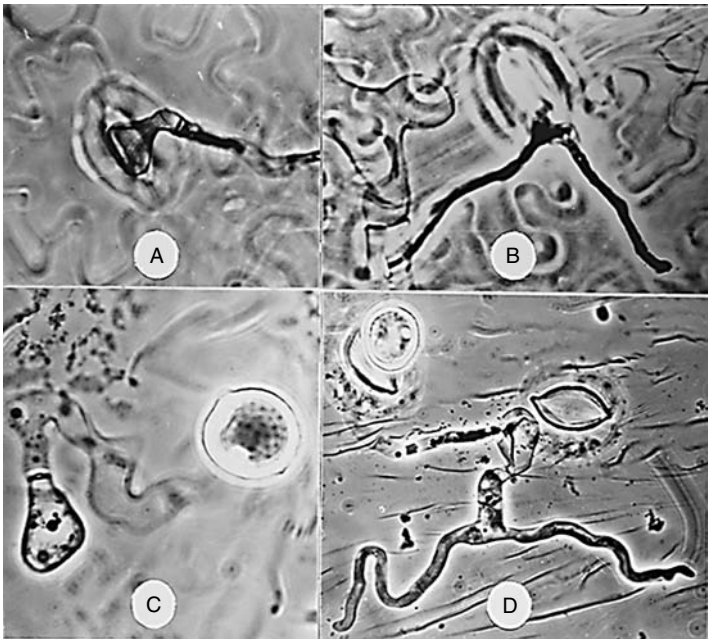


Figure 4.5 Diagram of infection structures developed from germinating urediospore of the rust fungus, *Puccinia antirrhini*. (a) Appressorium over stomata in snapdragon leaf. (b) Substomatal vesicle on the underside of leaf epidermis. (c) Appressorium on isolated leaf cuticle. (d) Substomatal vesicle and infection hyphae on isolated cuticle. (From Maheshwari et al. (1967). With permission of American Phytopathological Society.)

the haustorium occurs under the control of species- or even variety-specific signals and is also the site for the exchange of information between the host and the parasite. Mendgen and coworkers succeeded in isolating haustoria from rust fungus-infected broad bean leaves by separating them from tissue homogenates by affinity chromatography with the lectin, concanavalin A (Hahn and Mendgen, 1997; Voegele et al., 2001). The mRNA prepared from haustoria was used to construct a cDNA library of infection structures formed *in vitro* (Figure 4.7), which was screened by Northern (RNA) hybridization for genes specifically expressed in haustorium. Sequence analysis identified genes involved in nutrient uptake and vitamin biosynthesis. Using antibodies against the yeast hexose transporter as a heterologous probe and against a putative plant-induced amino acid transporter, nutrient transporters were immunochemically localized exclusively in haustorial plasma membrane of the bean rust fungus *Uromyces fabae* (Basidiomycotina), suggesting the uptake of sugar, and possibly of other nutrients (amino acids), occurs only through the haustorium. We need to understand how the fungal haustorium acts as a sink that effectively competes with the normal sink-source translocation pattern in plants. The specific localization of nutrient transporters in haustorium (Figure 4.8) suggests that the identification of host stimuli for haustoria differentiation will be crucial in designing conditions for the *in vitro* culture of obligately parasitic fungi on artificial media. Obligate parasites such as the white rust fungus *Albugo* (Straminipila), the powdery mildew fungus

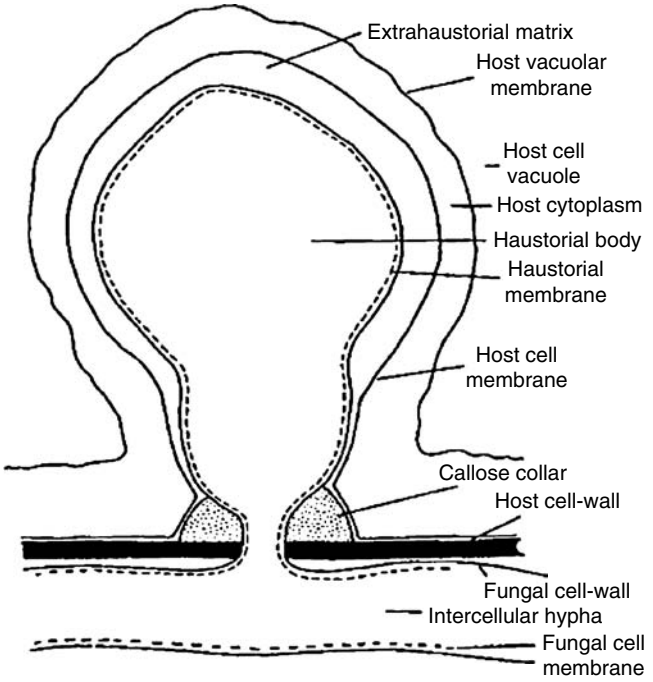


Figure 4.6 Diagram of a haustorium. The haustorium invaginates the host cell membrane. (From Ingold and Hudson, *The Biology of Fungi* (1973), Chapman and Hall. With permission of Kluwer Academic Publishers.)

Erysiphe (Ascomycotina) and the rust fungi *Uromyces* and *Puccinia* (Basidiomycotina) are among important pathogens of crop plants.

4.4.4 Production of Cutinase

Several fungi penetrate host tissue directly through the cuticle. The structural polymer of plant cuticle is cutin, a polyester composed of C_{16} and C_{18} fatty acids that covers the aerial parts of plants and acts as a barrier to direct penetration. However, on contact some fungi produce inducible cutinase enzymes that extracellularly degrade cutin by hydrolyzing the ester linkages. A role of cutinase in fungal pathogenesis was obtained by studying the infection of papaya fruit by *Mycosphaerella* (Dickman et al., 1989). The fungus infected papaya fruit only if the skin of the fruit was mechanically breached before applying spores. However, transformation of a *Mycosphaerella* sp. (Ascomycotina) that lacked the ability to produce cutinase with the *Fusarium* (Anamorphici) cutinase gene, resulted in the production of cutinase in transformants. The severity of lesions in transformant was correlated with the cutinase activity and including antibodies against *Fusarium* cutinase with spore inoculum prevented infection.

4.4.5 Production of Toxins

After entry into a host, some fungi secrete metabolic products of diverse structures called toxins which suppress resistance, alter the pattern of development (stunt or stimulate

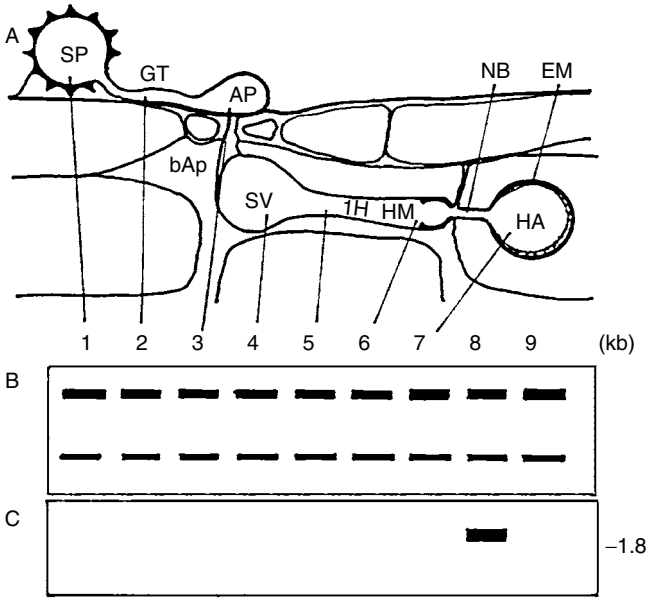


Figure 4.7 Specific expression of hexose transporter gene in haustoria. (A) Diagram of infection structure in vertical section of leaf tissue. SP, urediospore; GT, germ tube; AP, appressorium; SV, substomatal vesicle; IH, infection hypha; HM, haustorial mother cell; HA, haustorium. (B) Ethidium bromide stained DNA in denaturing agarose gel. (C) Northern blot of gel in B. (Adapted from Voegele et al. (2001).)

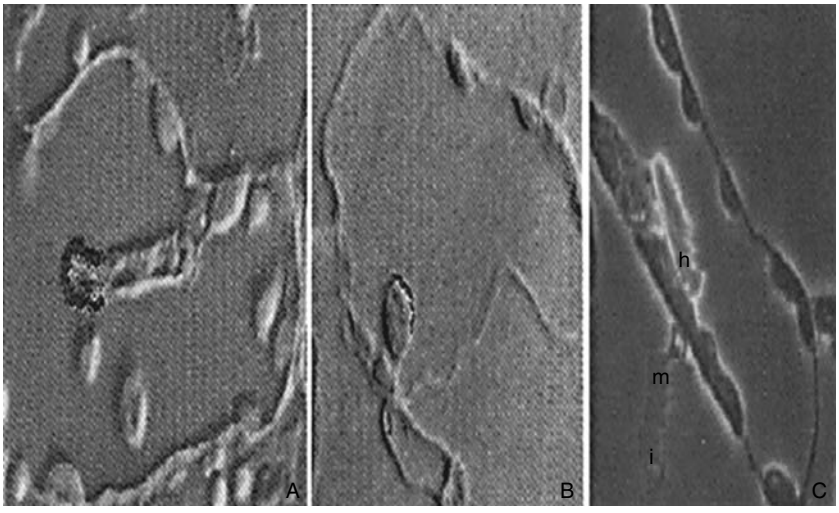


Figure 4.8 Specific localization of amino-acid transporter in haustoria of a rust fungus by immunofluorescence mmicroscopy. Early (A) and fully developed (B and C) haustorium in bean leaf infected with the rust fungus, *Uromyces fabae*. h, haustorium; m, haustorium mother cell; j, intercelluilar hypha. The haustorial neck is marked by arrow. (From Hahn et al. (1997). With permission of the American Phytopathological Society.)

growth), cause necrotic lesions (cell death) or wilting of plants. The toxins are low molecular weight secondary metabolites (e.g., polyketide, cyclic tetrapeptide, sesquiterpene epoxide) that are not essential for normal growth and reproduction of fungi. For its growth the fungus uses the leakage of electrolytes, sugars and amino acids from the killed host cells. A common procedure in work with toxins is to grow the parasite in culture and see if purified extracts from the cultures applied at concentrations that could be reasonably expected in the diseased plant reproduce some of the disease symptoms caused by the parasite.

Victorin is a toxin produced by *Cochliobolus heterostrophus* (Ascomycotina), the causal agent of Victoria blight of oats active at picomolar concentrations and exhibits the same specificity toward oat genotypes as the fungus (Navarre and Wolpert, 1999). It is therefore a host-selective plant toxin and is a cyclized pentapeptide of 814 Dalton. Leaves treated with victorin exhibit cleavage of a photosynthetic carbon-dioxide fixing enzyme, ribulose-1,5-bisphosphate carboxylase (Rubisco), and loss of chlorophyll. In addition, victorin-treated leaves show DNA laddering—cleavage into discrete sizes—characteristic of apoptosis or programmed cell death, suggesting that victorin causes premature senescence of leaves. *Mycosphaerella zeae-maydis* (Ascomycotina) produces a family of long-chain (C_{35} to C_{41}) polyketides, called T-toxin and PM-toxin, respectively. The T-toxin specifically affects mitochondria of cultivar carrying the Texas cytoplasmic gene introduced to simplify the production of hybrid varieties. However, mitochondria of normal maize (corn) are not affected by T-toxin. The binding of the toxin to a mitochondrial membrane protein causes pores to form in mitochondrial membranes, resulting in leakage of respiratory substrates, cessation of ATP production and cell death.

The identification of biosynthetic pathways of toxin production, the isolation of toxin non-producing mutants and the development of DNA-mediated transformation of pathogenic fungal species should provide critical proof for the role of toxins in pathogenesis (Desjardins and Hohn, 1997).

4.4.6 Detoxification of Saponins

Many plants constitutively produce triterpenoid, steroid or steroidal glycosylated compounds that are generally inhibitory to fungi (Figure 4.9). These are known by the general term *saponin* because of their soap-like properties, derived from the plant *Saponaria officinalis*, the extracts of which were once used to make soap (Osbourn, 1996). Saponins make complexes with membrane sterols, resulting in pore formation and leakage of cell constituents. The leaves and green fruits of the tomato plant contain high levels a steroidal glycoalkaloid, called tomatine (Figure 4.10). The pathogenecity of *Septoria lycopersici* (Anamorphici) on tomato plants was attributed to the production of a glycosyl hydrolase, tomatinase (Arneson and Durbin, 1967), which detoxifies tomatine by removing a single terminal glucose molecule by hydrolysis of a β,1-2 linkage. The targeted gene-disruption technique was used to test the role of saponins in pathogenecity. The root-infecting fungus *Gaeumannomyces graminis* var. *tritici* (Ascomycotina) causes the "take-all" disease of wheat and barley but is unable to infect oat because of its inability to detoxify the saponin avenacin A-1 that is present in oats. *G. graminis* var. *tritici* can, however, infect a wild oat species that did not produce avenacin A-1. The gene encoding avenacinase was cloned and used to disrupt avenacinase by site-directed mutagenesis of the genomic copy in var.

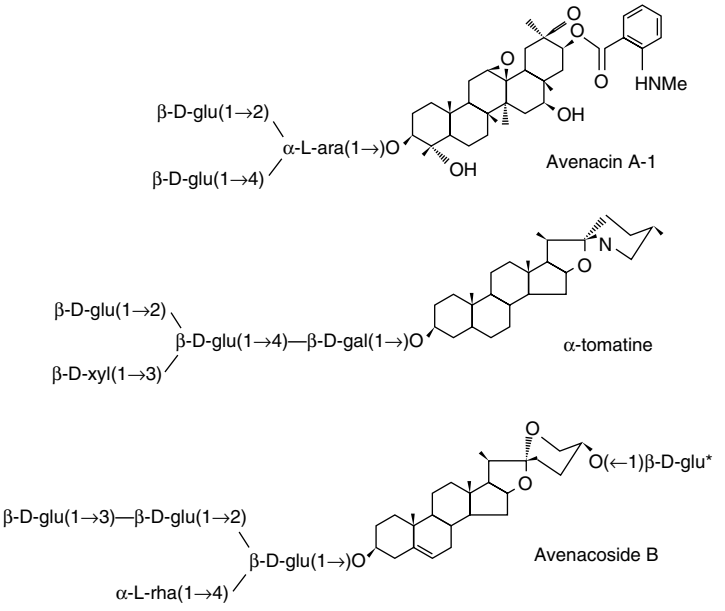


Figure 4.9 Chemical structures of saponins. Avenacin A-1 is a triterpenoid saponin in oat root. α-Tomatine is tomato steroidal glycoalkaloid. Avenaconaside B is an oat leaf saponin. These three saponins have a sugar chain attached to carbon-3 via oxygen. Avenaconaside B has an additional sugar moiety (β-D-glucose) attached via oxygen to c-26. Avenaconaside A differs from avenaconaside B in that it lacks the terminal β(1-3)-linked D-glucose molecule. The removal of C-26 glucose by the pathogen is indicated by an asterisk. (From Osbourn (1996).)

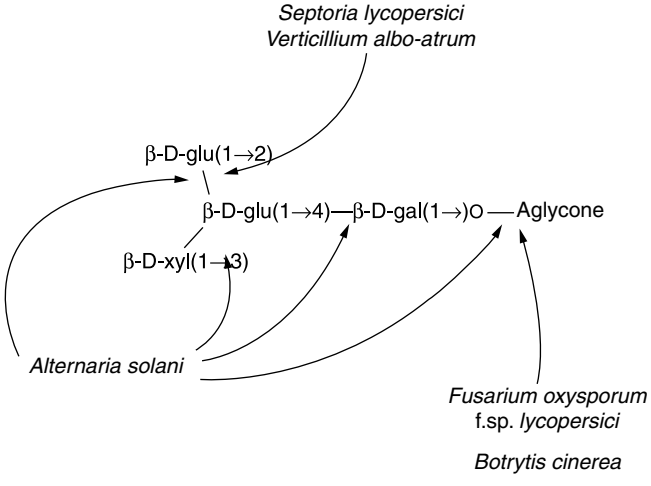


Figure 4.10 Detoxification of saponins by tomato pathogens. (From Osbourn (1996).)

avenae. The var. *avenae* transformants lost pathogenecity to oats but not to wheat. These examples suggest the role of saponin detoxifying enzymes in pathogenesis.

4.5 CONCLUDING REMARKS

In nature, resistance rather than susceptibility is the rule. The vast majority of fungi are strict saprophytes. The evolution of fungal phytopathogens has apparently proceeded from saprophytes to facultative parasites to obligate parasites. Physiological races of obligate parasites show the highest level of complexity. The apical growth of the hypha, its ability to perceive topographical signals and orient hyphal growth, its generation of mechanical force for entry through narrow openings, overcome host defense mechanisms by secreting enzymes capable of detoxifying plant secondary metabolites, stimulate nutrient leakage from infected cells and killing of host cells, secretion of toxic molecules that affect a fundamental biochemical process or neutralizes plant resistance or the production of enzymes capable of breaching structural barriers are among the attributes that makes a fungus a potential pathogen. Obviously one of these is not sufficient to make a virulent pathogen without the other capabilities. Likewise, a fungus may possess one or more of these properties but not be pathogenic.

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Part III

Model Fungi in Research

Chapter 5

Neurospora: A Gateway to Biology

In 1843, a luxuriant growth of a pink-orange fungus was observed on bread in the bakeries of Paris. This fungus was subsequently recognized as a common contaminant of bakeries and it came to be known as the pink bread mold. On September 1, 1923, an earthquake followed by fire struck Tokyo. The strange sight of a pink-orange growth that developed on almost all burnt trees and vegetation amazed the residents. Examination showed that the orange colored growth was due to the profuse production of conidia by a fungus. The fungus was named *Oidium aurantiacum*, later changed to *Monilia sitophila*. In 1927, Shear and Dodge discovered the sexual phase of the fungus in cultures grown in the laboratory and renamed the genus as *Neurospora* because it produced ascospores with neuron-like striations. *Neurospora* is the best studied of all fungi and is regarded as a model of all microbes (Davis and Perkins, 2002). This chapter describes some aspects of its history, life style, attributes and its contributions to biology.

5.1 HABITAT, LIFE STYLE AND LIFE CYCLE

Neurospora (Figure 5.1) is frequently seen growing on stubbles of sugar cane after the canes have been harvested for milling and the agricultural field is burnt (Pandit and Maheshwari, 1996) to clear the trash of the cut leaves. In addition to the pink-orange type, a yellow-colored *N. intermedia* is found in Asia on maize cobs which have been discarded on road-sides, parks or railway tracks after the roasted kernels are eaten by residents. Another species, *N. discreta*—a species thought to be infrequent, limited mainly to Ivory Coast and Papua New Guinea—was found growing beneath the bark of trees damaged by wildfires in western North America, including Alaska (Jacobson et al., 2003). In the laboratory, the vegetative growth of all species of *Neurospora* occurs satisfactorily on a simple nutrient medium between 20 and 40°C. The reason for substrate preferences or differences in geographic distribution of species in nature is a mystery but could be due to their preferences for certain types of substrates for sexual reproduction. For example, *N. crassa* and *N. intermedia* show good fertility in media containing sucrose, whereas the *N. discreta* crosses are infertile on sucrose medium but satisfactorily reproduce on media containing filter paper cellulose. The yellow strains of *N. intermedia*, unlike the orange strains, reproduce poorly in media containing sucrose though they are very fertile on corncobs. A general observation is that conditions that favor sexual reproduction are different from those that favor asexual reproduction.

Figure 5.1 *Neurospora* growing in nature. (A) Mass of orange conidia erupting through cracked epidermis of a burnt sugar cane stump in an agricultural field. (B) Conidia erupting at a node through the openings created by burning of the adventitious roots. A pencil was placed as a size marker. (See color insert following page 140.)

The linear growth rate of Neurospora—up to 5 mm per hour—is one of the fastest among the fungi. The growth rate is easily measured in a "race tube" in which mycelium growth on an agar surface is confined to one dimension, allowing the position of the advancing mycelium to be marked at regular intervals (see Figure 11.6). Morphologically similar strains can be physiologically different. For example, the heterothallic species *N. crassa* and *N. sitophila* are comprised of strains of the opposite mating types, referred to as *A* and *a*, which can be crossed (mated) to form structures (fruiting bodies) called *perithecia* that enclose asci bearing meiotically formed ascospores. In general, the species produce eight ascospores of which four each are of the two mating types. Barbara McClintock, who discovered mobile genetic elements (transposable elements) in maize and a discovery for which she received the Nobel Prize, also studied *Neurospora*. In 1945, using acetoorcein staining and light microscopy, McClintock showed that *Neurospora* has seven chromosomes (haploid number $n = 7$, meaning it has one chromosome of each type and therefore one copy of every gene). The chromosomes are numbered based on their length and this numbering based on their size does not correspond to the numbering of the linkage groups. The term *linkage group* refers to an order of linked genes whose linkage has been determined on the basis of recombination frequency. The numbering of linkage group is in the chronological order of their discovery. Linkage groups correspond to chromosome numbers but may not correspond to the numbering of chromosomes based on the basis of their size. For example: LGI = chromosome 1, LGII = chromosome 6,

LGIII = chromosome 3, LGIV = chromosome 4, LGV = chromosome 2, LGVI = chromosome 6 and LGVII = chromosome 7. The chromosomes can be separated by pulsed field gel electrophoresis (PFGE). The total DNA of *N. crassa* is 43 megabases; the largest and the smallest chromosomes are 10.3 and 4 megabases, respectively.

The life cycle of *Neurospora crassa* based on laboratory-grown cultures is shown in Figure 5.2. The sexually (meiotically) formed ascospores of *Neurospora* are constitutively dormant propagules that can survive in soil for several years. The activated ascospores germinate and form mycelium which produces two types of asexual (mitotically

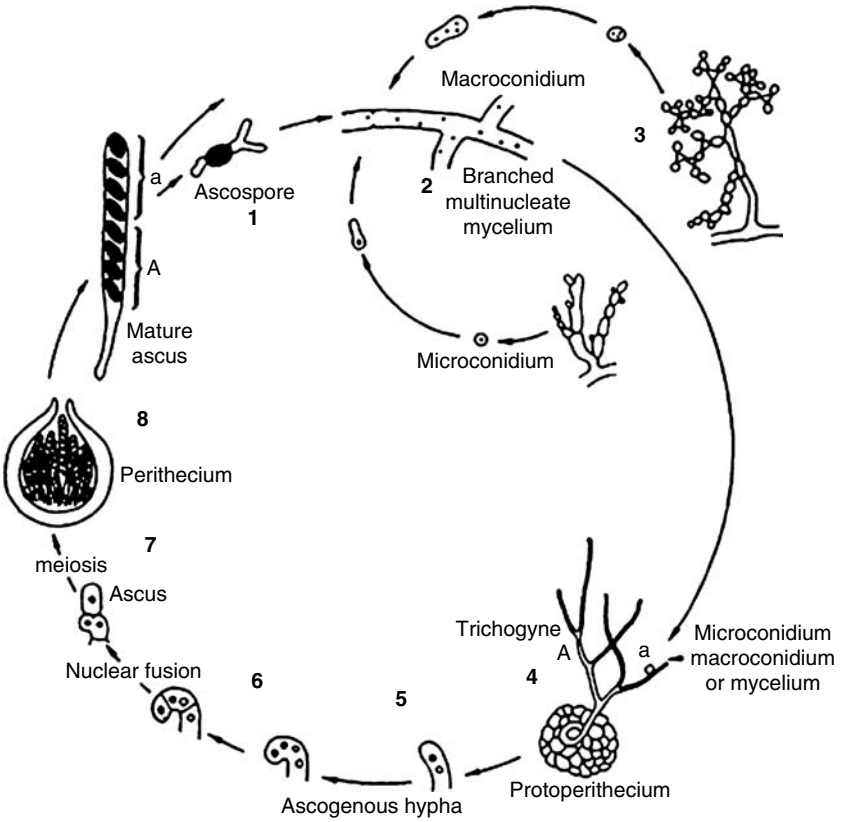

Figure 5.2 Life-cycle of *Neurospora crassa*. The ascospore (1) germinates after chemical or heat activation and forms mycelium. Asexual reproduction occurs by formation of mitotically-formed inconspicuous, uninucleate microconidia (2) or 2-3 nucleate orange-colored macroconidia (3) which are dispersed. Sexual cycle begins with coiling of hyphae around ascogonial cells from which a trichogyne projects out formation of protoperithecium (4). The trichogyne picks a nucleus of opposite mating type from microconidia, microconidia or hyphal (stages 5 and 6). Nuclear fusion (stage 7) leads to formation of asci containing 8 haploid ascospores of the two mating types, *A* and *a*. From Perkins et al. (2000). With permission from Elsevier.

derived) spores. Under conditions of high-sugar and nitrogen availability, the fungus produces powdery pink-orange (macro) conidia that are 5 to 9 μm in diameter and contain 2 to 6 nuclei, whereas under low-nutrient conditions the fungus mainly produces inconspicuous, uninucleate microconidia that are 2.5 to 3.5 μm. The microconidia function as male cells and are formed simultaneously with structures called *protoperithecia* that are formed by the coiling of hyphae around ascogonial cells, one cell of which acts as the female gamete. DNA sequence analysis shows that the two alleles of the mating type locus, *mat A* and *mat a*, or *A* and *a*, have highly dissimilar DNA sequences and for this reason they are termed *idiomorphs* (Metzenberg and Glass, 1990). Mating occurs only between strains of *A* and *a* mating types—that is, between individuals that are sexually compatible. A slender hypha called the *trichogyne* projects out from the protoperithecium and is attracted toward macro- or microconidium of the opposite mating type, indicating that recognition of a mating partner is based on pheromone and pheromone receptor. The nucleus, picked up by this trichogyne (Figure 5.3), migrates to the ascogonium where fertilization occurs. In the laboratory, crosses are made by adding macroconidia (because these are obtained easily) from one culture to a culture of the opposite mating type that has been pre-grown in a medium with low nitrogen and carbon to induce the formation of protoperithecia. The haploid nuclei of the opposite mating type fuse in a hook-shaped structure called a *crozier*. The diploid zygote nucleus does not undergo divisions; rather, it immediately undergoes meiosis. The not-readily noticeable ascospores are formed at a different time from the striking conidial phase and were therefore missed for a long time.

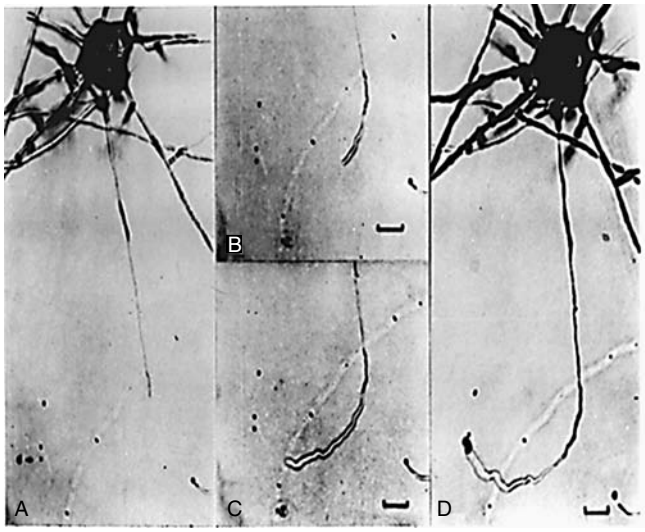

Figure 5.3 Chemotactic growth of trichogyne of *Neurospora crassa*. (A) Microconidia (male cells) were placed at lower left side. (B–D) Curvature of trichogyne is a visible evidence of production of a pheromone by conidia. Reprinted with permission from G.N. Bistis (1981), *Mycologia*, Vol. 63, Number 5. © The Mycological Society of America.

In *Neurospora*, the four haploid nuclei, produced from a single meiotic division, divide mitotically to produce eight nuclei that are individually sequestered into eight oval-shaped cells called the *ascospores* and are enclosed in a single elongated cell, called the *ascus*. As this development is going on, the protoperithecium darkens and forms a flask-shaped structure called the *perithecium* with an opening, called the *ostiole*. The mature, dormant ascospores are shot out through the ostiole. The ascospores (haploid cells) germinate when conditions are appropriate and produce a mycelium that contains haploid nuclei that have only one chromosome of each type and therefore one copy of each gene. The fungus displays the alternation of haploid and diploid phases but the haploid phase persists for a long time and is the dominant phase. The haploid mycelium produces the characteristic pink-orange colored conidia by which the fungus is easily recognized in nature and collected.

Approximately 65% of all *Neurospora* that have been collected globally are comprised of one species called *N. intermedia* (Turner et al., 2001). Its life history was determined based on observations of the fungus on burnt sugar cane in agricultural fields and from simulated experiments in the laboratory (Pandit and Maheshwari, 1996). Sampling of propagules in air in sugar cane fields did not reveal ascospores, discounting the theory of their aerial dissemination. However, virtually all samples of soil from the sugar cane field, after it had been heated to 60°C for 30 to 45 min—a treatment that kills conidia—yielded *Neurospora*. In the field-cultivated sugar cane, *Neurospora* colonies were on the burnt stumps or the stubbles in contact with the soil, suggesting that ascospores present in soil infect the burnt cane. To confirm this, cane segments were planted in soil into which reciprocal mixtures of ascospores and of conidia of wild type (al^+, orange) and a color mutant (*al*, albino/white) had been mixed. The cane segments were burnt in the laboratory, simulating the conditions in the field. The phenotype of *Neurospora* colonies that developed on the cane segments was that of the genotype of the ascospores that had been mixed into the soil, confirming that the heat-resistant ascospores in soil initiate infection of burnt cane. In nature, the activation of dormant ascospores is brought about by heat generated by burning, or by furfural—a compound produced on heating xylose that occurs as a constituent of the polysaccharide xylan in the plant cell walls.

The life cycle of *Neurospora* illustrates that most fungi produce spores both asexually and sexually, i.e., fungi are pleomorphic. Environmental conditions determine when, where and how reproduction will occur. On the burnt sugar cane, conidia formed in the sugar-rich cane tissue and their production continued until the sugars had become depleted. The production of astronomical numbers of conidia externally and their constant dissemination by wind is seemingly wasteful but it is the fungus' strategy of removing sugars from plant tissue and to create a nutritional environment for sexual reproduction to occur (Pandit and Maheshwari, 1966). The depleted nutrient conditions favors the development of protoperithecia and microconidia, i.e., for sexual reproduction to occur. Further, so long as sugar is available, the absorptive mycelium ramifies inside the tissue and forms closely packed lateral aggregates of conidiophores (sporodochium) beneath the epidermal tissue. The growth pressure of sporodochium causes the epidermis to separate from the ground tissue, thereby creating tissue pockets in which microconidiophores and protoperithecia, a biological solution necessary to ensure humid conditions and attract microfauna for effecting fertilization by transmitting microconidia to trichogyne. The sexual phase is relatively inconspicuous, develops at a different location and may appear unrelated, and was therefore unnoticed for a long time. The occurrence of morphologically distinct asexual and sexual phases at different times due to their differing environmental requirements illustrates that in fungi the condition for asexual and sexual reproduction are often quite different.

5.2 MEIOTIC EVENTS

B.O. Dodge and C.C. Lindegren perceived *Neurospora* as a marvelous organism for genetic analysis as the four products of a single meiosis are packaged as ascospores in a single row in the order of their formation in ascus (usually called a *tetrad*, although in *Neurospora* the meiotic division is followed by a mitotic division; hence, each of the four meiotically produced ascospores are duplicated as sister ascospores, thus an *octad*). The ascospores are large enough, measuring approximately 29×15 μm, to be dissected manually under a dissecting microscope and their phenotypes and genotypes to be determined. This procedure, called *tetrad analysis*, allows tracing the order of the events in meiosis. For example, by crossing a mutant strain that affects ascospore color, it can be determined simply by visual examination of the asci in opened perithecia (Figure 5.4) whether the separation of parental alleles occurred in the first meiotic division without crossing over (first division segregation) or in the second division as a result of cross over between the gene pair and centromere. As an example, the 4:4 ratio of mating types (*A* or *a*) or of ascospore color marker (*cys-3*) in the octad demonstrates that alleles separate during meiosis, validating Mendel's First Law of Segregation. (In genetic nomenclature for *Neurospora*, the name of a gene is generally abbreviated to the first three letters. For example, the mutant allele *cys* refers to cysteine

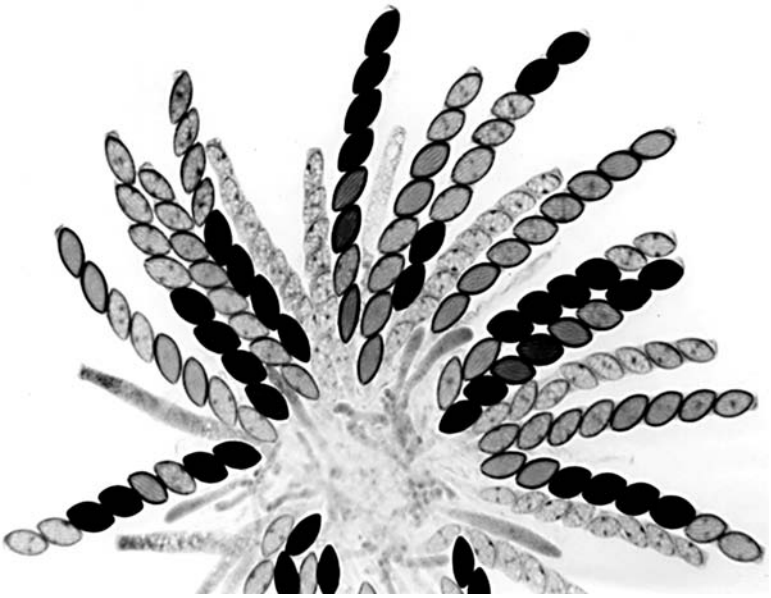

Figure 5.4 Opened perithecium of *Neurospora crassa*. Photomicrograph of a rosette of maturing asci from a cross of wild-type (+) × *cys-3* mutant (*m*) allele that affects cysteine biosynthesis and results in unpigmented (white) ascospores. Because the final division is mitotic, contiguous sister ascospores have the same genetical constitution. One ascus at top center and two asci at upper left show four black : four white first-division segregation. The remaining mature asci show second division segregation patterns (2+, 2*m*, 2+, 2*m*) resulting from crossing over between *cys-3* and centromere. Photo courtesy of Namboori B. Raju.

while the superscript "+" is used to distinguish the wild allele from the mutant allele). In a cross of a wild *N. crassa* with pink-orange carotenoid pigment, i.e., al^+ and aerial mycelium (col^+) to a double mutant albino (white, impaired in carotenoid biosynthesis) and colonial (restricted growth in the form of a colony), four types of progeny are produced in equal proportion: al^+ col^+ (wild type with orange, aerial mycelium), *al col* (albino colonial), al^+col (pink-orange colonial) and *al* col^+ (albino, spreading mycelium), in conformity with Mendel's Second Law of Independent Assortment. From a *Neurospora* cross, a significant number of sexual progeny can be grown in small tubes and analyzed in about four weeks, providing considerable savings both in space and time than would be possible with either a pea plant or a fruit fly. *Neurospora* has been the main source of information about the basic recombination mechanisms in eukaryotes. It is an ideal classroom material for teaching of microbiological and genetic methods.

Tetrad analysis demonstrated that meiotic recombination by crossing-over between linked loci on the same chromosome occurs after chromosome replication between chromatids and not whole chromosomes. In a cross ($AB \times ab$) where genes *A* and *B* are linked, with alleles *a* and *b*, the appearance of two parental and two recombinant products (a tetratype) led to the conclusion that crossing-over occurs after chromosome replication between non-sister homologous chromatids at the four-strand stage (Figure 5.5). Had it

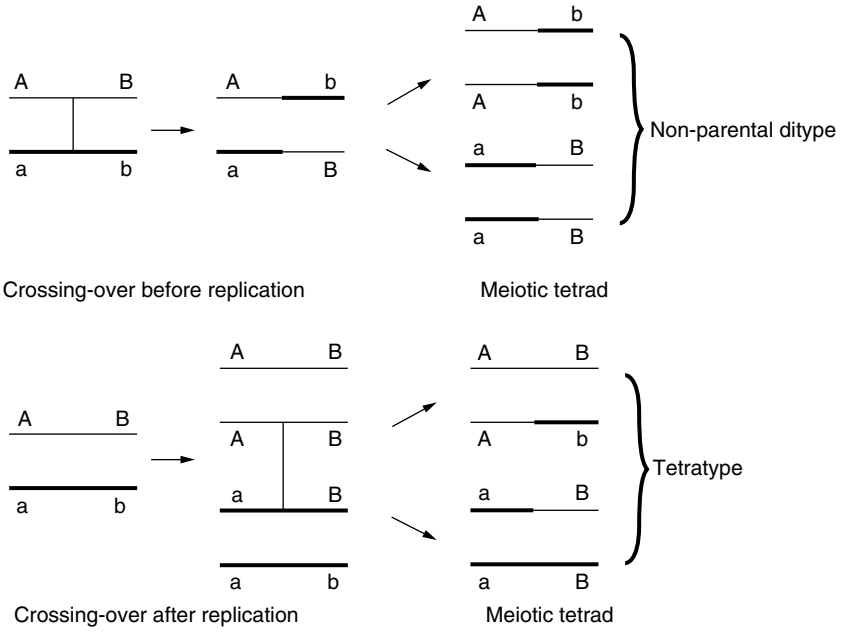

Figure 5.5 Meiotic recombination between linked loci. Crossing-over (shown as a vertical line between homologous chromosomes) between genes *AB* and alleles *ab* occurs at the four-strand stage; that is, after homologous chromosomes had each duplicated into two chromatids. Products of meiotic division will divide by mitosis and the mature ascus will have eight ascospores.

occurred before replication, no tetratypes would have been found. *Neurospora* began to be used for understanding recombination mechanisms (Figure 5.6). Infrequently, a small proportion of aberrant 6 + : 2 mutant and 2 + : 6 mutant asci instead of normal 4 + : 4 mutant asci were detected (Mitchell, 1955). Such aberrant segregation ratios were explained by gene conversion according to which allele at a locus converts corresponding allele at the same locus into its own type. This observation provided clues on molecular models of crossing over that could account for gene conversion (Holliday, 1964).

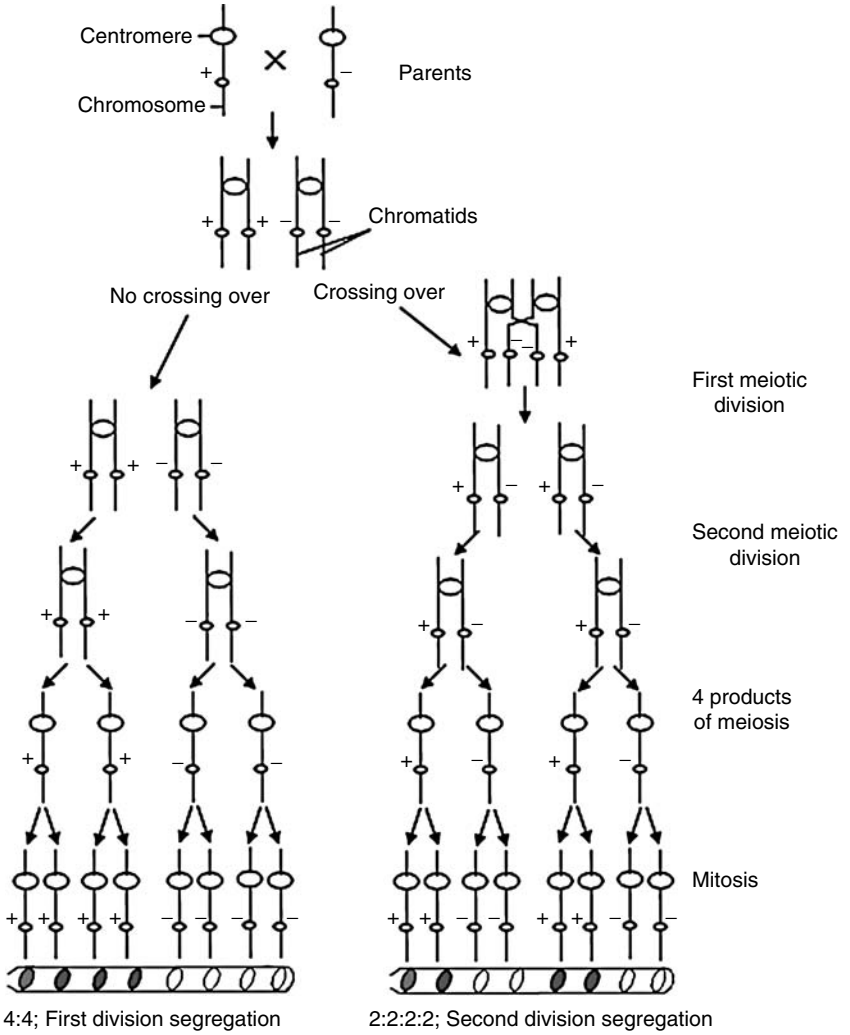

Figure 5.6 Scheme of first- and second-division segregation in *Neurospora*. Segregation of two + and – alleles can be deferred to the second division of meiosis, a phenomenon known as "second-division segregation."

5.3 GENE MAPS

In *Neurospora* the ascospores are ejected successively—the asci elongate, one at a time protrude through the opening in the perithecium and shoot ascospores in groups of eight and then retract. Thus, if the time of their collection is kept short, well-separated groups of tetrads (i.e., products of single meiotic events) can be collected (Figure 5.7).

Neurospora is the first organism for which techniques were developed for detecting and analyzing chromosomal rearrangements in which segments between chromosomes become exchanged, or deleted, or inverted (Figure 5.8), either spontaneously, by UV irradiation or by DNA-mediated transformation. These are types of mutations. The rearrangement of chromosome structure is manifested as visually defective ascospores in shot octads (Perkins, 1994). The defective ascospores are white while non-deficiency ascospores are black. To identify the nature of chromomosome rearrangements, the strain to be tested is crossed to a normal strain in a petri dish. After ten days, the perithecial surface is briefly inverted over a slab of water agar to collect spontaneously shot groups of eight ascospores (unordered tetrads) and examined under a binocular microscope to score defective octad (black:white) classes as 8:0, 6:2, 4:4, 2:6 and 0:8. The frequency of the 4:4 type is diagnostic of the type of translocation, whether insertional or reciprocal (Figure 5.9). Unordered octads are used in many aspects of meiotic genetics, including the measurement of cross-over frequencies based on the proportion of asci of parental ditypes, tetratypes and nonparental ditypes. From the data, the distance between a gene and centromere can be determined and gene maps constructed.

To facilitate the mapping of a newly discovered gene, a linkage tester strain, *alcoy*, having three reciprocal translocations was developed in which six linkage groups are represented as T(IR; IIR) *al-1*, T(IVR; VR) *cot-1* and T(IIIR; VIL) *ylo*. The symbol *T* refers to translocation, the numbers refer to linkage groups and the symbols *R* and

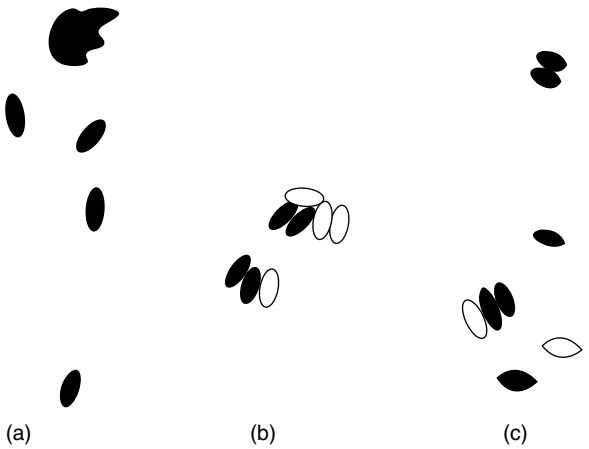

Figure 5.7 Spontaneously shot ascospore octads from a *Neurospora crassa* cross insertional translocation × normal. (a) All normal (black). (b) Four normal : four defective (white). (c) Six normal : two defective. From Perkins (1974). With permission of Genetics Society of America.

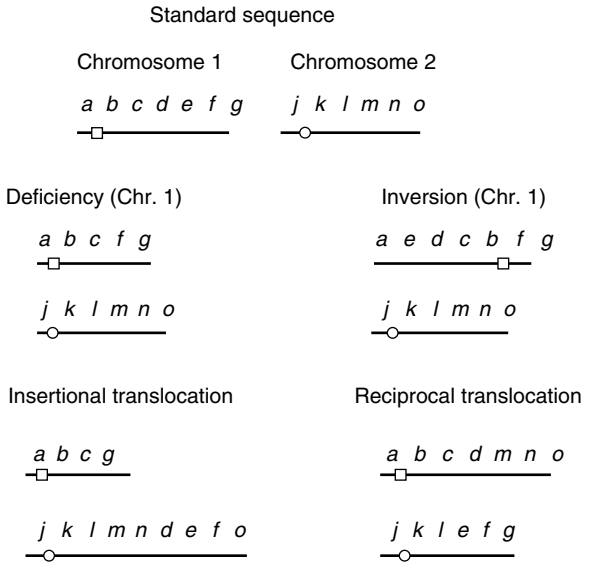
Standard sequence
Chromosome 1
Chromosome 2
a b c d e f g
j k l m n o
Deficiency (Chr. 1)
a b c f g
j k l m n o
Inversion (Chr. 1)
a e d c b f g
j k l m n o
Insertional translocation
a b c g
j k l m n d e f o
Reciprocal translocation
a b c d m n o
j k l e f g

Figure 5.8 Common types of chromosomal rearrangements found in *N. crassa*. From Davis (2000). With permission from the Oxford University Press.

L refer to the right and left arms of the linkage groups. The compound chromosomes have visible genetic markers near the junction point: *albino-1* (*al*, white color), *colonial temperature sensitive-1* (*cot*, button-shaped colony at 34°C) and *yellow* (yellow color). Segregation of these markers among progeny helps in the detection of the linkage of an unknown gene to any of these markers. The use of this linkage tester facilitates the rapid mapping of new mutations on a particular chromosome, thereby greatly reducing the time required to find the linkage of an unknown gene than if it is to be found by crossing the unknown to each of the seven linkage group tester strains. Consider the example of a mapping mutant senescent strain that ceases to grow in four to five subcultures. A cross of a wild strain with conidia from a senescent strain produced half wild type (+) and half senescent progeny, showing the segregation of a single gene pair according to Mendel's principle and identifying the "death" phenotype to be due to a single gene, called *senescent* (*sen*). A single cross of the mutant senescent to *alcoy* gave an essentially random recombination of *sen* with *al-1* (41%), eliminating linkage groups (LG) I and II from consideration. On the other hand, *sen* showed 22% recombination with *cot-1*, indicating that *sen* is either on LG IV or V. A follow-up cross of *sen* to a strain having *cot-1* marker on the normal chromosome produced parental and recombinant classes in nearly equal frequency (48 and 52%), showing that *sen* is not on LG IV. From a cross of *sen* to a strain containing two mutant markers, *cycloheximide-2 resistant* and *albino-3* in LGV, the frequency of recombination between *cyh-2* and *sen* (37%) and between *sen* and *al-3* (11%), *sen* was positioned between *cyh-2* and *al-3* (Figure 5.10).

Nearly a thousand genes have been mapped in *N. crassa* (Perkins et al., 2000). It has the most saturated genetic map of all fungi. The gene maps show that functionally related

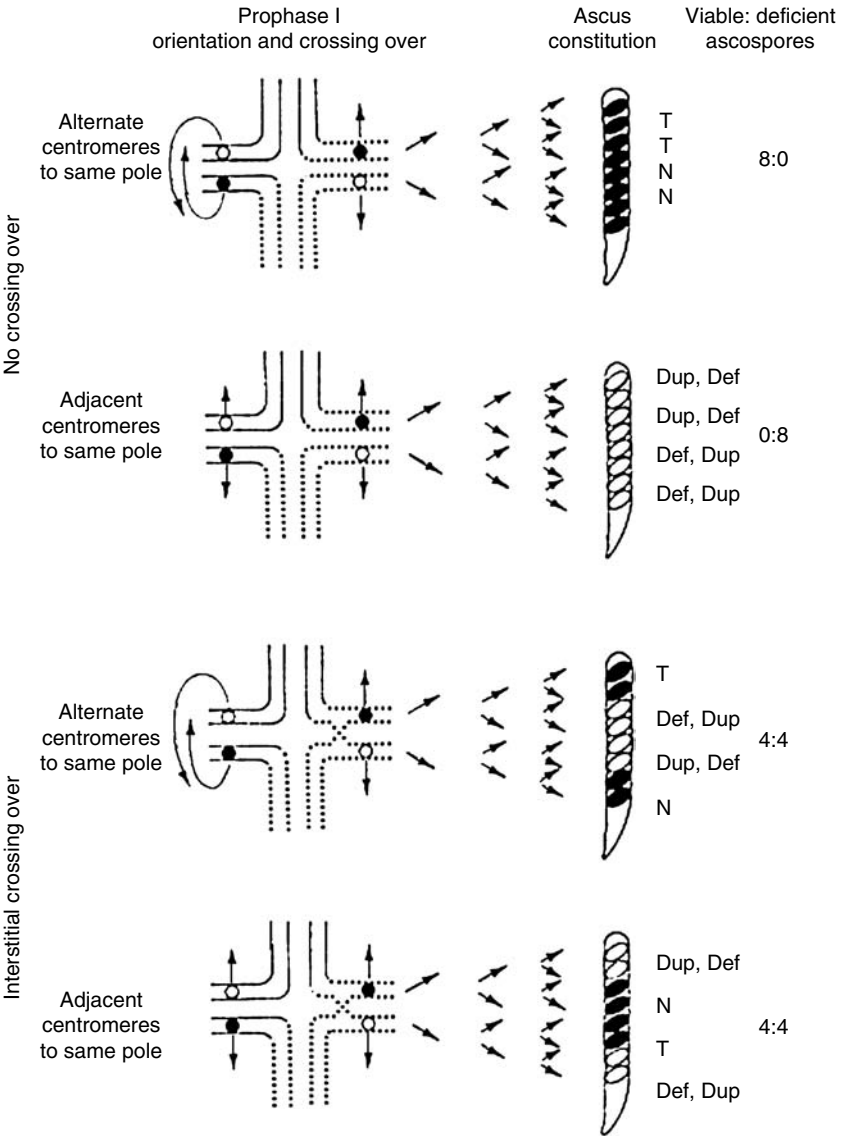

Figure 5.9 Segregation pattern for a cross reciprocal translocation (black centromeres) × normal sequence (white centromeres). From Perkins (1974). With permission from Genetics Society of America.

genes in the same biosynthetic pathway are not generally linked or clustered. Another fact learned from the mapping work is that crossover frequencies, while reproducible in crosses with the same two parents, vary widely in crosses with parents of different genetic backgrounds. The gene maps showed also that most genes related to the sexual cycle are not linked to the mating type locus (idiomorphs).

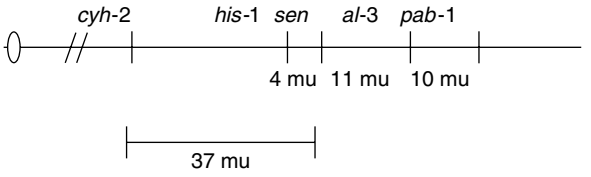

Figure 5.10 A partial gene map of linkage group VR. Abbreviations of genes: *cyh-2*, cycloheximide resistant-2; *his-1*, histidine-1; *sen*, senescent; *al-3*, albino-3; *pab-1*, para-aminobenzoic acid-1. One map unit (mu) = 1% recombination between gene loci.

5.4 ONE GENE-ONE ENZYME HYPOTHESIS

In 1941, George Beadle and Edward Tatum, respectively a geneticist and a biochemist, wished to determine whether mutations can lead to nutritional requirements. For this they required an organism whose life cycle and genetics was worked out and that could be grown on a simple medium of known chemical composition so that any mutation-induced nutritional deficiency could be identified simply by supplying the fungus with a known metabolite. The organism of choice was *Neurospora crassa* as it requires only a simple minimal salt and sugar medium with only biotin supplement. Beadle and Tatum irradiated *Neurospora* conidia with x-rays to induce mutations. The irradiated conidia were used to cross a culture of the opposite mating type and the ascospores that formed were tested for mutant phenotypes (Figure 5.11). Strains were selected on the basis of

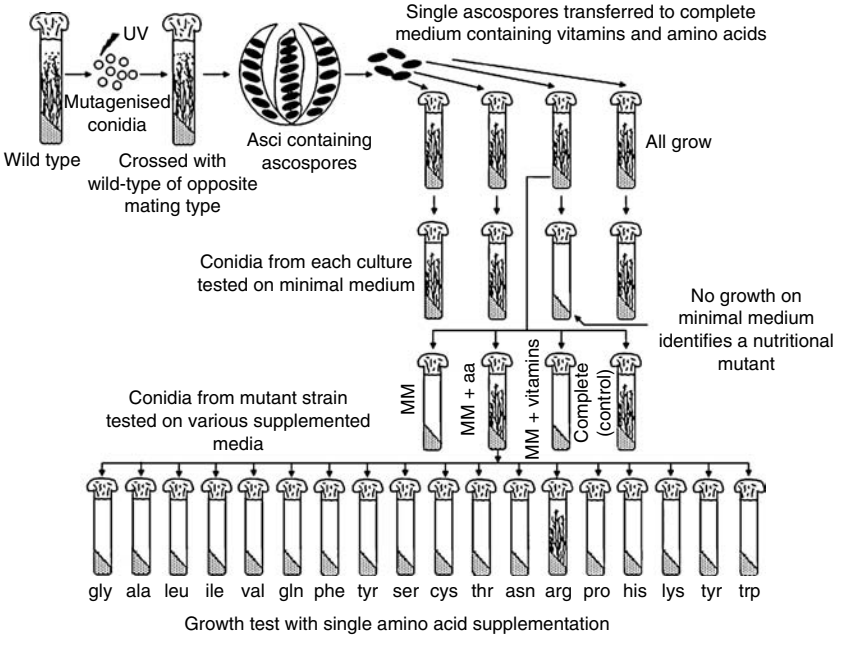
UV
Mutagenised conidia
Wild type
Crossed with wild-type of opposite mating type
Asci containing ascospores
Single ascospores transferred to complete medium containing vitamins and amino acids
All grow
Conidia from each culture tested on minimal medium
No growth on minimal medium identifies a nutritional mutant
Conidia from mutant strain tested on various supplemented media
MM
MM + aa
MM + vitamins
Complete (control)
gly ala leu ile val gln phe tyr ser cys thr asn arg pro his lys tyr trp
Growth test with single amino acid supplementation

Figure 5.11 The procedure used by Beadle and Tatum for production and identification of auxotrophic mutants in *Neurospra crassa*. MM, minimal medium; aa, amino acid.

differential growth response in media lacking or containing a single chemical compound. In each case, the auxotrophic mutation (a mutation that results in the requirement of any of the metabolic end products) was inherited as a single-gene mutation, i.e., when crossed to a wild (original standard) strain it gave a 1:1 ratio of the mutant and the wild-type progeny.

As an example, a set of mutant strains required arginine to grow on minimal medium. By a gene mapping procedure, Beadle and Tatum found that these mutants mapped into three different locations on separate chromosomes, even though all could grow if the same supplement (arginine) was added to the medium. However, three mutants, *arg-1*, *arg-2* and *arg-3*, differed in their response to related compounds, ornithine, citrulline and arginine. The *arg-1* mutants grew with supplementation of ornithine, citrulline or arginine. The *arg-2* mutants grew on either arginine or citrulline but not on ornithine. The *arg-3* mutants grew only when arginine was supplied. The results demonstrated that synthesis of arginine proceeds from ornithine through citrulline and that each mutant strain lacks only one enzyme. Based on the properties of the *arg* mutants of *Neurospora*, Beadle and Tatum proposed a sequence of reaction in which each reaction is controlled by a specific enzyme, identified by a specific gene (Figure 5.12).

This model, known as the one gene-one enzyme hypothesis, was inferred from the properties of mutant classes. It provided the first insight into the functions of genes, that each gene controls one specific enzyme. Nearly all mutants responded to single growth supplement, i.e., each mutant was blocked in a single biosynthetic pathway. The position of the mutational block in the biochemical pathway could be determined by growth tests, whether the accumulation of the last intermediate preceded the block or occurred after the block. There are enzymes encoded by more than one gene, however, the one gene-one enzyme hypothesis fits most genes. This work demonstrated that the mutant approach can be generally exploited to determine individual steps in metabolic pathways. In 1958, George Beadle and Edward Tatum were awarded the Nobel Prize (shared with Joshua Lederberg) for their discovery that genes act by specifying enzymes that catalyze definite chemical reactions. The discovery laid the foundation of biochemical genetics. Beadle (1966) recalled:

> During the course of this work Tatum's late father, Arthur Lawrie Tatum, then Professor of Pharmacology at the University of Wisconsin, visited Stanford. On this occasion he

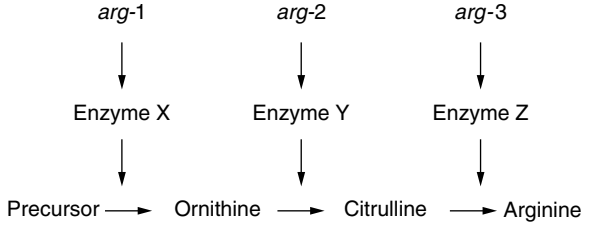

Figure 5.12 A biochemical pathway illustrating one gene-one enzyme concept. Metabolic sequences are identified by the intermediates formed and purifying the enzymes that catalyze their conversion.

called me aside and expressed concern about his son's future. "Here he is," he said, "not clearly either biochemist or geneticist. What is his future?" I attempted to reassure him—and perhaps myself as well—by emphasizing that biochemical genetics was a coming field with glowing future and there was no slightest need to worry.

5.5 MOLECULAR REVOLUTION

From the one gene-one enzyme hypothesis arose the concept of colinearity of gene and protein, i.e., the linear array of DNA nucleotide base sequence specifies the linear array of amino acids in the protein. Since the amino acid sequence of a protein was not yet known, Beadle and Tatum did not know the way in which genes determined enzymes. However, the biochemical genetic work initiated by *Neurospora* inspired workers using *E. coli* and yeast and was a starting point of a molecular revolution in biology. It was established that allelic forms of a gene specify structurally different forms of the same enzyme. Further, it was observed that certain pairs of mutants, deficient in the same enzyme, cooperated in diploid or in a heterokaryon to produce significant amounts of enzyme activity, i.e., the polypeptide chains from different mutants corrected each other's defects (allelic complementation). It was shown that many enzyme proteins contain more than one polypeptide chain, each specified by a different gene. The formation of active enzyme molecules was explained as molecular hybrids in which polypeptide chains from different mutants corrected each other's defects through effects on polypeptide conformation. These observations led to the modification of the one gene-one enzyme hypothesis to the one gene-one polypeptide hypothesis. The basic relationships between genes, proteins and phenotypes thus understood, attention turned to how the activities of enzymes are controlled. This led to the formulation of the operon model of control of gene activity from work done with *E. coli*. After the genetic basis of the enzyme structure was established, the work with *E. coli* led to investigations of the regulation of gene activity, stimulated by the lactose operon model of Jacob and Monod. The growth tests with the auxotrophic mutants of *Neurospora* started a molecular revolution in biology. "It became the preferred way to dissect complex biological systems such as embryonic development, cell division, the nature of sensory systems, and aging. Today, mutational analysis is the preferred way into a complex biological problem, especially as it provides access to the genes and protein players" (Horowitz et al., 2004).

5.6 REVELATIONS FROM GENOME SEQUENCE

To understand how filamentous fungi work and to understand their evolution means that one must have a complete genomic sequence whereby one can monitor gene expression and correlate it with metabolic and developmental phenotype. Because in the model eukaryotic microbes (yeast and *Neurospora*) most genes have been characterized based on mutant phenotypes, these were chosen for genome sequencing efforts in fungi (Galagan et al., 2003; Borkovich et al., 2004). The genome sequence of *Neurospora* was released in 2003 with the features of sequence given in Table 5.1. Although the *Neurospora* genome is, respectively, about 4 and 100 times shorter than those of fruit fly and man, the number of genes is high. The 43 megabase pair genome encodes about 10,000 genes, nearly twice as many genes as in the yeast *Saccharomyces cerevisiae*. Many *Neurospora* genes lack homologues in yeast and may be specific for filamentous lifestyle. Approximately 14%

Table 5.1 Features of Neurospora crassa genome

Size	38,639,769 bp
Chromosomes	7
% G + C	50
Protein-coding genes	10,082
Protein-coding genes (> 100 amino acids)	4200
tRNA genes	424
5S rRNA genes	74
Percent coding genes	44
Average gene size	1673 bp (481 amino acids)
% Protein-coding sequences	
Similar to known sequences	13
Conserved hypothetical proteins	46
Predicted proteins (no similarity to known sequences)	40

Adapted from Galagan et al. (2003).

of *Neurospora* genes match proteins in either plants or animals, suggesting a closer relation of filamentous fungi to higher forms than to yeast. A surprise revelation was that *N. crassa* has homologs of phytochrome, required for red-far red sensing in plants. The fungus shares genes with complex organisms that measure time (biological clock). The fungus has methylated sequences, relics of transposons. Another revelation is that though a saprophyte, *Neurospora* possesses genes for virulence factors and enzymes for plant cell wall digestion required for fungal pathogenesis, raising the possibility if this fungus, hitherto considered to be a saprophyte, could be an endophyte or an opportunistic plant parasite. Further similarity in the two types of fungi is suggested by genes for secondary metabolite production; for example, gibberellin biosynthesis and polyketide metabolism are present. The fungus has mechanisms that inactivate duplicated (repeated) sequences in the sexual cycle (Chapter 9). Many methylated DNA sequences, generally associated with duplicated genes are present, indicating relics of transposons.

5.7 CONCLUDING REMARKS

A technical requirement of an organism that could be cultured in a defined medium led to the adoption of *Neurospora crassa* as an experimental organism with which to address the question of what controls metabolic reactions. The resolution of this question by using induced mutants attracted the imagination of several workers on genetical approach to biological problems. *N. crassa* played a role in connecting genetics and biochemistry, heralding biochemical genetics. More than a half-century since its introduction in research, *Neurospora* continues to be used in studies on hyphal mode of growth (Chapter 1), nuclear interactions in heterokaryons (Chapter 4), mutational and epi-genetical gene silencing mechanisms (Chapter 9), biological clocks (Chapter 11), speciation (Chapter 13), nuclear and plasmid-based senescence phenomenon (Chapter 14) and in many others, such as the meiotic drive elements that distort genetic ratios (Raju, 1996; Perkins, 2003). The biological knowledge of *Neurospora* from the molecular to population level has made *Neurospora* shift from model to an ideal reference organism—the fungus in which fundamentals are established and to which other fungi are compared (Davis and Perkins, 2002).

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Chapter 6

Yeast: A Unicellular Paradigm for Complex Biological Processes

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> In short order, yeast became a supermodel, challenging even *E. coli* in the visibility of its contributions.
>
> *Rowland H. Davis*

6.1 INTRODUCTION

Since antiquity, yeast has been domesticated unwittingly or purposefully for the conversion of grape juice into wine by a process called fermentation. In the eighteenth century, the French chemist Antoine Lavoisier (1734–1794) showed that sugar was transformed into ethanol during fermentation. Theodor Schwann (1810–1882) and Charles Cagniard-Latour (1977–1859) microscopically examined fermentation mixtures and advanced the view that the "force" that drove fermentation is "a mass of globules that reproduces by budding" and is a consequence of the growth of yeast—an idea that was quickly rejected by the influential German chemist Justus von Liebig, who maintained that the murkiness in fermenting liquid was not due to a living organism. Based on controlled experiments, chemical analyses of broth and microscopic examinations of the sediment from successful and "diseased" fermentation vats, the versatile French scientist Louis Pasteur (1822–1895) concluded that yeast cells did not spontaneously arise from fermenting liquid but from preexisting cells. He identified yeast as the causative agent of alcoholic fermentation. The brewer's yeast *Saccharomyces cerevisiae* has today become a supermodel—"an organism that reveals and integrates many diverse biological findings applying to most living things" (Davis, 2003). A number of investigators, distributed in approximately 700 laboratories around the world, have joined hands to make this fungus (though rather atypical) a model of all model organisms. Its advantages for the study of physiology and eukaryotic gene

[1]I wish to thank Morgen Kilbourn of CuraGen Corporation for generating figures.

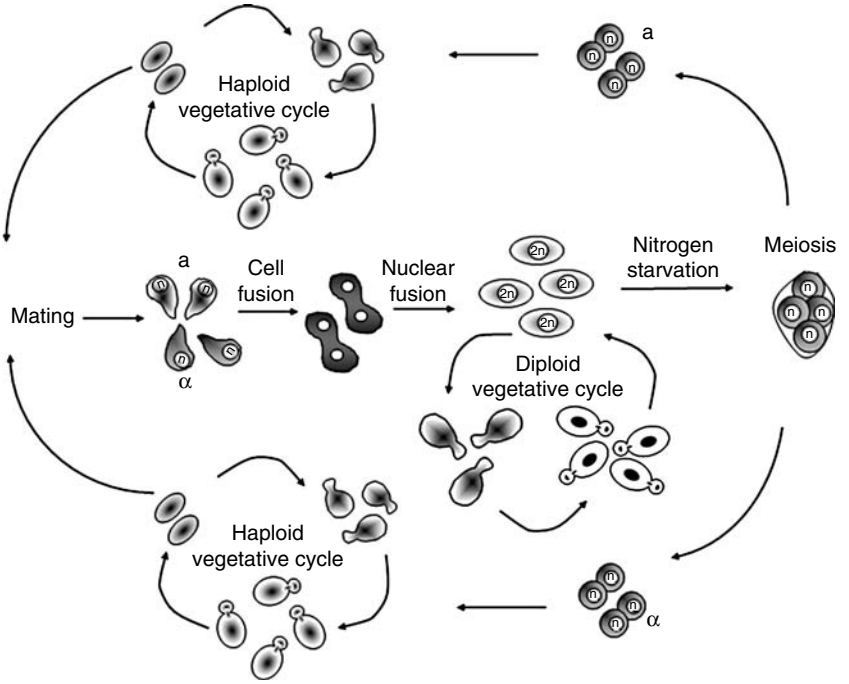

Figure 6.1 Life cycle of *Saccharomyces cerevisiae*. Both haploid and diploid cells multiply by budding. Diploid (2*n*) cell undergoes meiosis to form four haploid (*n*) cells, which are enclosed in a cell called ascus.

functions are: (1) its unicellular nature, making it a eukaryotic counterpart of *E. coli*; (2) its amenability for mass culture in a simple minimal medium with a doubling time of about an hour; (3) the stability of its haploid and diploid phases (Figure 6.1); (4) the ease of generating and detecting mutants, including conditional-lethal mutations for study of indispensable gene functions; (5) availability of a large diversity of mutant stocks; (6) its growth under both anaerobic or aerobic conditions, making it ideal for study of mitochondrial biogenesis; (7) its small genome, the smallest of any eukaryote; (8) the highly efficient cloning of genes by simple complementation of mutant genes; (9) the homologous integration of transforming DNA allowing disruption, deletion or replacement of a gene; and (10) its two-hybrid method to generate a protein-interaction map for a system biology modeling of multicellular organisms (Giot et al., 2003). This chapter gives some remarkable examples of using yeast in the study of biological processes in the eukaryotes and the likely further developments.

6.2 MOLECULAR MECHANISMS OF DNA REPLICATION AND CELL DIVISION

One of the most significant contributions of *Saccharomyces cerevisiae* (the brewer's, baker's or budding yeast) and the fission yeast *Schizosaccharomyces pombe* to biology is in the understanding of eukaryotic cell cycle. The mitotic cell division is accomplished by a temporal sequence of events in which the cell first duplicates the DNA (S-phase), followed

by precise separation of the sister chromatids into daughter nuclei (M-phase), leading finally to the separation of the two daughter cells. Each phase is separated from the preceding phase by an interval of time. The gap G1 is the time interval between mitosis and the initiation of new DNA synthesis; G2 is the gap between completion of DNA synthesis from the initiation of mitosis. In the late sixties, Leland H. Hartwell took a genetic approach to understand the biochemical basis behind the orderly sequence of events of the mitotic cycle. He capitalized on the genetic advantages of *S. cerevisiae* to generate cell division cycle (*cdc*) mutants (Hartwell et al., 1974). The *cdc* mutants were conditional temperature sensitive alleles (*ts* alleles), which could be stably maintained by growing the mutant cells at a permissive temperature of 23°C and exhibited the mutant phenotype of cell cycle arrest only at 37°C. The growth-arrested *cdc* mutants could be observed under a light microscope to determine accurately the position within the cell cycle at which they were arrested. The mapping of the cell cycle position with growth is possible in yeast by observing the ratio of the size of the bud (daughter cell) to the mother (Figure 6.2). The technique used by Hartwell for generating yeast *cdc*-mutants is shown in Figure 6.3.

Early genetic work with yeast accelerated the understanding of the eukaryotic cell cycle, first by forging a link between the events in the cycle with specific genes and second by establishing that the orderly sequence of events during cell cycle is a result of biochemical dependency, meaning that a prior event needed to be completed before the initiation of the

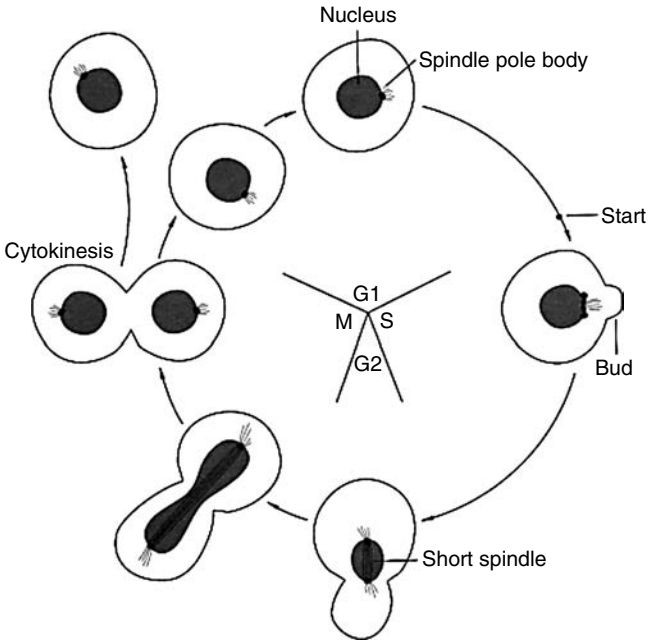

Figure 6.2 The *S. cerevisiae* cell cycle. The shape of a cell shows its position in its division cycle. The position START within G1 is the point at which the cell is committed to complete the cell cycle. The bud emerges at the beginning of S-phase and enlarges during G2 and M. The spindle pole bodies in yeast are embedded on the nuclear membrane. Yeast, like other fungi, has a closed mitosis—the nuclear envelope never breaks down. Reproduced with permission from Watson, J.D., Gilman, M., Witkowski, J. and Zoller, M. (1992), *Recombinant DNA*. © Scientific American Books.

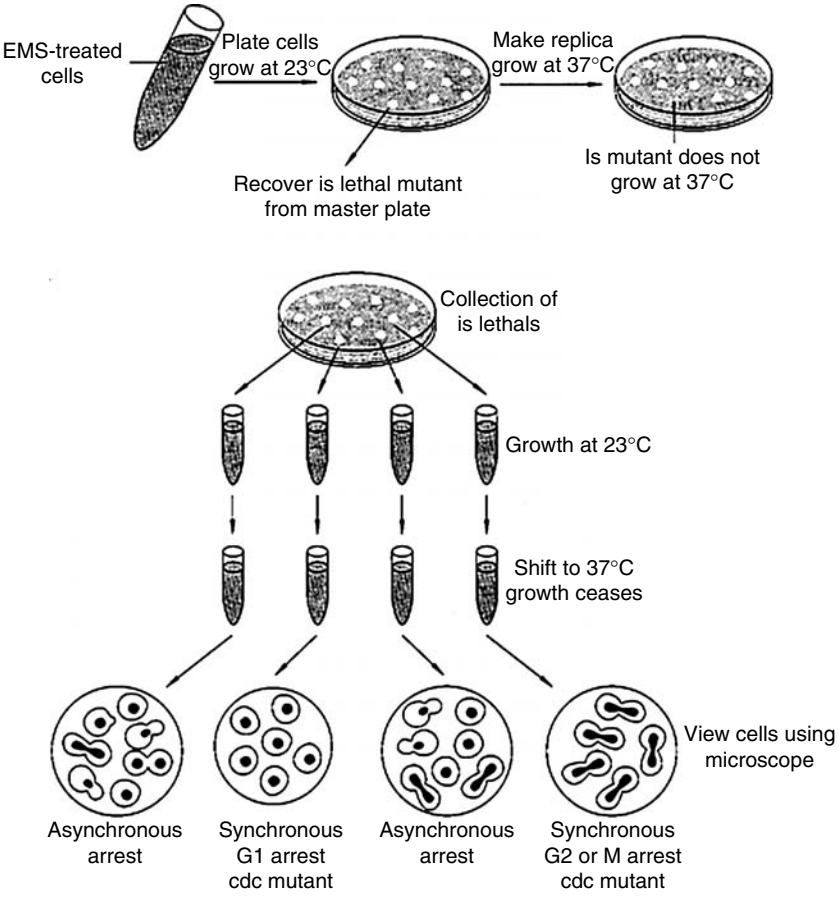
EMS-treated cells
Plate cells grow at 23°C
Make replica grow at 37°C
Recover is lethal mutant from master plate
Is mutant does not grow at 37°C
Collection of is lethals
Growth at 23°C
Shift to 37°C growth ceases
View cells using microscope
Asynchronous arrest
Synchronous G1 arrest cdc mutant
Asynchronous arrest
Synchronous G2 or M arrest cdc mutant

Figure 6.3 Isolation of *cdc* mutants. Cells, mutagenized with the DNA-methylating agent ethyl methanesulfonate (EMS), were grown at 23°C and then spread on agar at 23°C (the permissive temperature). The colonies that were formed were replica-plated and grown at 37°C (the non-permissive temperature). Individual colonies that failed to grow at 37°C were noted as temperature sensitive (*ts*) mutants. The *ts*-mutants from the master plate were grown at 23°C, shifted to 37°C for a few hours and examined by microscopy. Cells in most cultures arrested at random points in the cell cycle (asynchronous arrest) but some cells arrested at the same point of the cycle (synchronous arrest). The latter class of cells was called *cdc* mutants. Reproduced with permission from Watson, J.D., Gilman, M., Witkowski, J. and Zoller, M. (1992), *Recombinant DNA*. © Scientific American Books.

next. Finally, the availability of the *cdc* mutants allowed the cloning and identification of the genes regulating cell cycle by complementation as described in the next section.

6.2.1 *cdc* Genes

In all, 67 genes in the *Saccharomyces* genome database are annotated as *cdc* genes, of which 32 genes are directly involved in cell division. The rest regulate cell polarity and

bud growth without taking part directly in the process of cell division. Many of these 32 genes have been cloned by complementation (see Chapter 8). For example, *cdc28* mutation in *S. cerevisiae* arrests growth of cells at a point "Start" in G1, when the cell commits to enter the cell cycle. To clone this gene, a *cdc28 ts* mutant is transformed with a genomic library from wild (*CDC28*) yeast strain, the cells plated and incubated at non-permissive temperature (37°C) and the rare colonies in which *CDC28* gene was incorporated survived. (We shall follow the convention of naming a gene with three letters and a number. For *S. cerevisiae*, the wild-type alleles are written in uppercase letters in italics and mutant recessive alleles are written in lowercase letters in italics). The plasmid-carrying *CDC28* gene is recovered, sequenced and from the DNA sequence the encoded protein is identified as a protein kinase, an enzyme that transfers the terminal phosphate group from ATP onto a serine/threonine residue of another protein. Cloning of several *cdc* genes revealed that many of them are protein kinases whereas others are phosphatases (enzymes that remove phosphate groups from protein substrates). Paul Nurse showed that the human *CDC2* kinase was able to complement the loss of function mutation in yeast (Lee and Nurse, 1987), underscoring the evolutionary conservation of the yeast and the human kinase. In the meantime, work with sea urchin eggs by Tim Hunt led to identification of a 45 kd protein, whose levels in dividing cells fluctuated at a regular interval, that he called cyclin. Biochemical analysis in different organisms reveals that binding of cyclin to the protein kinase regulated the enzyme activity. The first of two important conclusions from these studies was that the cyclical nature of eukaryotic cell cycle is controlled by phosphorylation/dephosphorylation of cyclin-dependent kinases (CDKs) and by the targeted degradation of cyclin protein as shown in Figure 6.4. The second important conclusion was that the mechanism of cell cycle regulation is highly conserved in all organisms from yeast to human. Leland Hartwell, Paul Nurse and Tim Hunt received the Nobel Prize in 2001 for their contributions to understanding the fundamental process of cell division.

6.3 BUD GROWTH AND POLARITY

A basic problem in development is to understand the principles by which multicellular organisms determine the time and correct positions of new cells generating shape (patterning). This is the principle of *polarity*, seen at the level of an organism in the organization of head-and-foot structure of humans and animals, in the shoot-and-root system of plants, and so on. At the cellular level, polarity is observed in the plane of cell division as in the case of the *Fucus* zygote, where division plane of the nucleus in the rhizoid cell lies perpendicular to that in the thallus cell; in the organization of the nerve cells with dendrites at one end and axon at the other end; and in the structure of transporting epithelial cells lining our stomach and intestine with an apical and a basolateral end that separates two distinct compartments. The concept of polarity is equally prevalent in fundamental biological processes such as embryogenesis, where asymmetrical cleavage of the fertilized egg creates cells that follow distinct fates, or during communication between immune cells such as B and T-lymphocytes or during neurogenesis, where nerve cells are actively guided so that they reach and synapse at specific regions in the brain. Studies in the last four decades have revealed that the underlying mechanism behind all manifestations of polarity in unicellular or multicellular organisms lies in the generation of cellular asymmetry and a conserved mechanism involving cytoskeletal reorganization guides this process (Nelson, 2003).

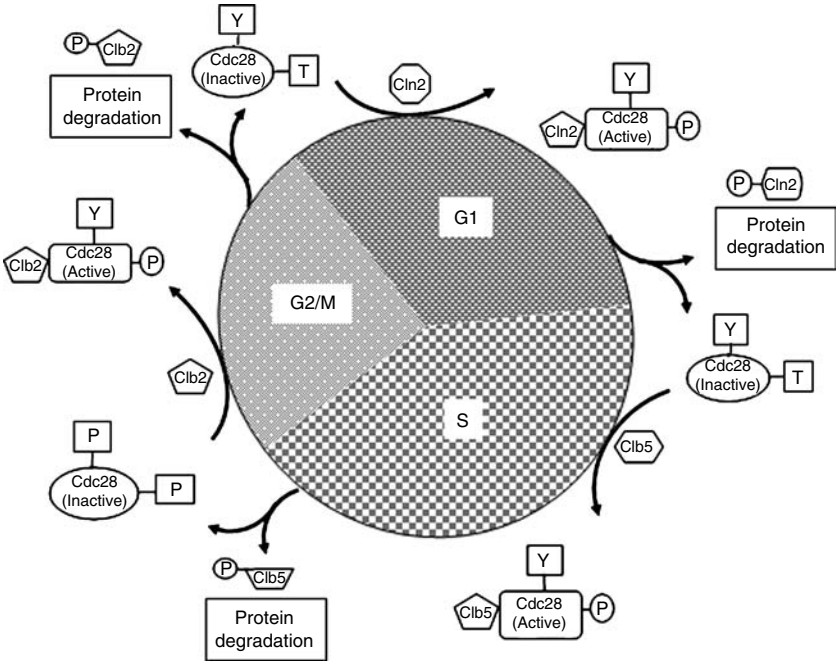

Figure 6.4 A biochemical model of cell cycle regulation in eukaryotes. Each phase of the cycle is regulated by the timely expression of a distinct cyclin protein. Entry of cells into mitosis is controlled by successive phosphorylation by kinases and dephosphorylation by phosphatases. The yeast cyclin-dependent kinase *CDC28p* is activated by phosphorylation of threonine-18 (T-18) and inhibited by phosphorylation of tyrosine-19 (Y 19). *CLN2p* commits the cell into START by activating *CDC28*. Once activated, *CDC28p* phosphorylates *CLN2p* resulting in dissociation of the complex. *CDC28* is inactivated by dephosphorylation and phosphorylated *CLN2p* is degraded. *CLB5* and *CLB2* proteins initiate S-phase and M-phase respectively. The end of M-phase is marked by rapid degradation of *CLB2* and dephosphorylation of *CDC28* kinase.

The budding and the fission yeast are attractive systems to understand cellular asymmetry at the molecular level. First, *S. cerevisiae* shows polarized growth at every cell division by taking a decision where to produce the bud. Second, the complex process is genetically tractable. The budding pattern is easily observed by staining yeast cells with a fluorescent dye calcofluor, which fluoresces after binding to cell wall chitin. Bud scars are especially rich in chitin and fluoresce brightly (Figure 6.5).

At every division cycle, *S. cerevisiae* selects the site of a new bud in a spatially distinct pattern (Freifelder, 1960). Haploid ***a*** or α cells choose bud sites in an axial pattern in which mother and daughter cells bud adjacent to their prior mother-bud junction. On the other hand, diploid *a* or α cells choose sites in a bipolar pattern in which mother cell buds either adjacent to the last daughter or at the pole opposite the last daughter. The daughter, however, always buds at the pole opposite its mother. The two distinct patterns of budding are shown in Figure 6.5.

In the original screen (Chant and Herskowitz, 1991), haploid yeast cells were mutagenized and plated on soft agar plates. Mutants defective in axial budding pattern were

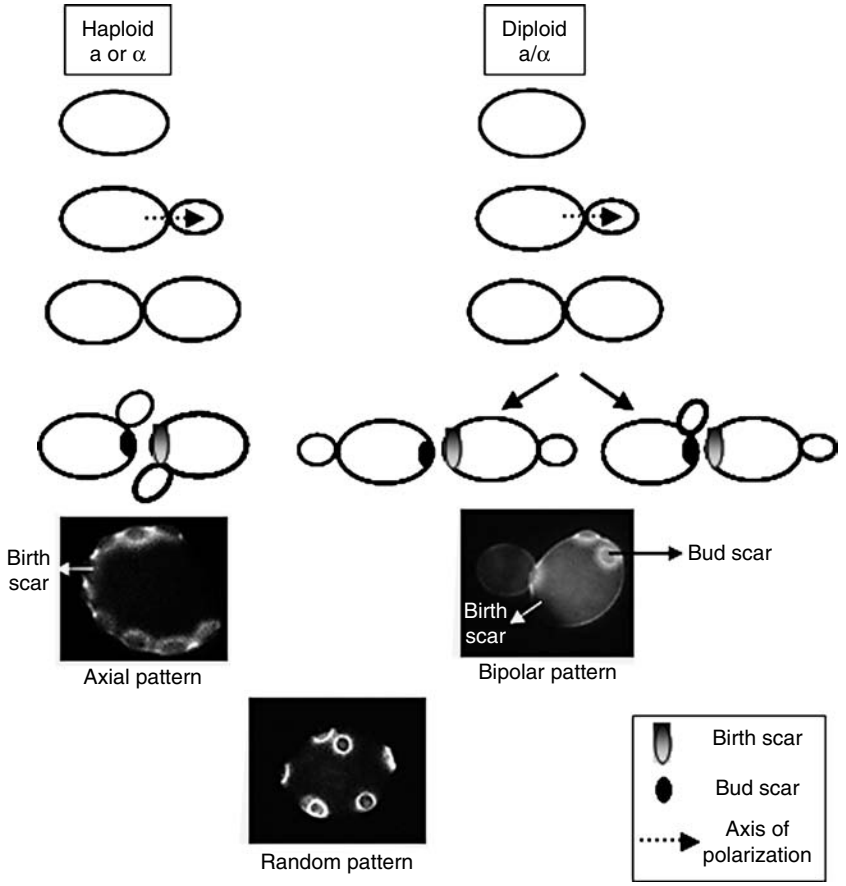

Figure 6.5 Bud site selection in *Saccharomyces cerevisiae*. The pattern of budding is characterized by the orientation of the bud scars with respect to the birth scar. In calcofluor staining, the birth scars appear as unstained areas whereas the birth scars are brightly fluorescent. In axial pattern the bud scars always lie juxtaposed to the birth scar and to each other forming a continuous line of scars. In bipolar budding, the bud scars are seen opposite to the birth scar and also adjacent to it as shown in the figure. In random pattern, the bud scars are seen arranged randomly on the yeast cell.

selected by observing microcolonies formed after the mother cell has undergone one to three cell divisions under a microscope. The pattern of budding is revealed by the arrangement of the daughter cells with respect to the mother cell in each microcolony. The original genetic screen identified four genes (*BUD1*, *BUD2*, *BUD3* and *BUD4*) required for the specification of the axial budding pattern. A fifth gene *BUD5* was identified by molecular approaches (Chant et al., 1991). Analysis of the function of the genes in haploids and diploids revealed that *BUD1*, *BUD2* and *BUD5* are required by both types of cells to select bud sites at correct positions, whereas mutation in *BUD3* and *BUD4* affected only haploid cells. Further work from various laboratories identified other genes that distinctly affected bud site selection in haploids and diploids. The mechanism of bud-site selection follows a two-step process. First, the yeast cell integrates intrinsic spatial information to define the site for the growth of the

new bud. Proteins that persist at the site of the previous bud from one cell division to the next produce these spatial cues. Localization experiments using GFP-tagged proteins support the persistence of *BUD3*, *BUD4* and *BUD10* at previous bud sites after each division cycle. Once the site is selected, the next step involves recruitment of a GTP-binding protein *BUD1* and its regulators *BUD2* and *BUD5* to the site previously marked by *BUD3*, *BUD4* and *BUD10*, leading to the localized activation of *BUD1*. It is believed that active *BUD1* activates *CDC42* locally by interacting with its GTP-exchange factor *CDC24*. Active *CDC42* then induces actin cytoskeletal reorganization by regulating the activity of actin-binding proteins. As a result of cell polarization, the protein transport machinery delivers membranes and components of cell wall biosynthesis at the site of the growing bud.

Bud site selection in diploids is mediated by a different group of landmark proteins *BUD8* and *BUD9* that mark the poles of the cell by recruiting *RAX2p*, which persists at the poles for many generations and is believed to activate *CDC42p* via *BUD1p*. In the absence of *BUD8* or *BUD9*, *RAX2p* fails to localize correctly, causing the diploid to bud in a random pattern. A schematic representation of the molecular machinery that guides the axis of polarization during budding is shown in Figure 6.6.

Although it may appear that generation of polarity in unicellular yeast is different from that in epithelial cells of multicellular organisms, the basic core mechanism of

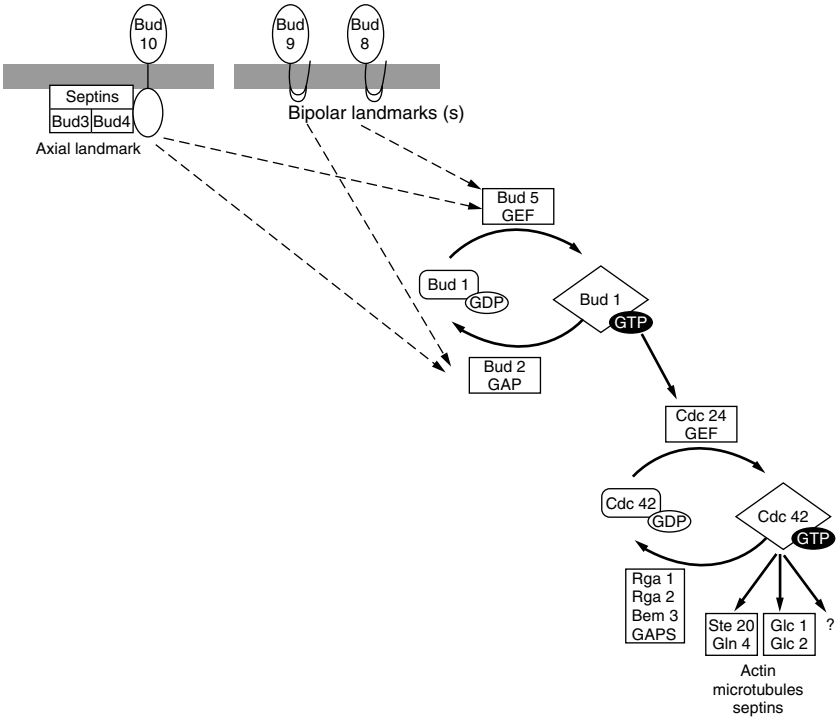

Figure 6.6 Mechanisms that generate cell polarity during bud-site selection in *S. cerevisiae*. The spatial landmark proteins on the cell surface guide the cellular machinery to build the axis of polarization through the GTP-binding proteins *BUD1* and *CDC42*. Reproduced with permission Figure 4a from Chant, J. (1999), *Ann. Rev. Cell Dev. Biol.* 15, 365–391. © Annual Reviews Inc.

organizing the cytoskeleton using *CDC42* and actin-binding proteins arose very early during evolution to be used universally by all eukaryotic organisms.

6.4 MATING AND SIGNAL TRANSDUCTION CASCADE

Yeast cells exist in ***a***- and α-mating types (Chapter 8). Mating of cells of opposite mating types is coordinated by the release of small peptide hormones (pheromones). The **a**-cells produce a 12-amino acid peptide, the **a**-pheromone, and responds to the 13-amino acid peptide *a*-pheromone, produced by a-cells. The a-cells on the other hand bind the **a**-pheromone produced by the **a**-cells. Reception of the pheromone signal triggers a series of events that include changes in cell shape and arrest of cell growth. How these events are triggered by the pheromone signal is of general interest because signaling events must occur in unicellular organisms seeking nutrients in the surrounding environment, or in a fungal pathogen searching for an entry point into a host plant, or in a higher organism responding to growth factors, hormones, neurotransmitters and other sensory input.

The pheromone signaling is the most well-characterized eukaryotic signaling pathway. The hunt for genes in this pathway was facilitated by a selection scheme based on the growth arrest of cells in response to pheromone signaling as schematically depicted in Figure 6.7.

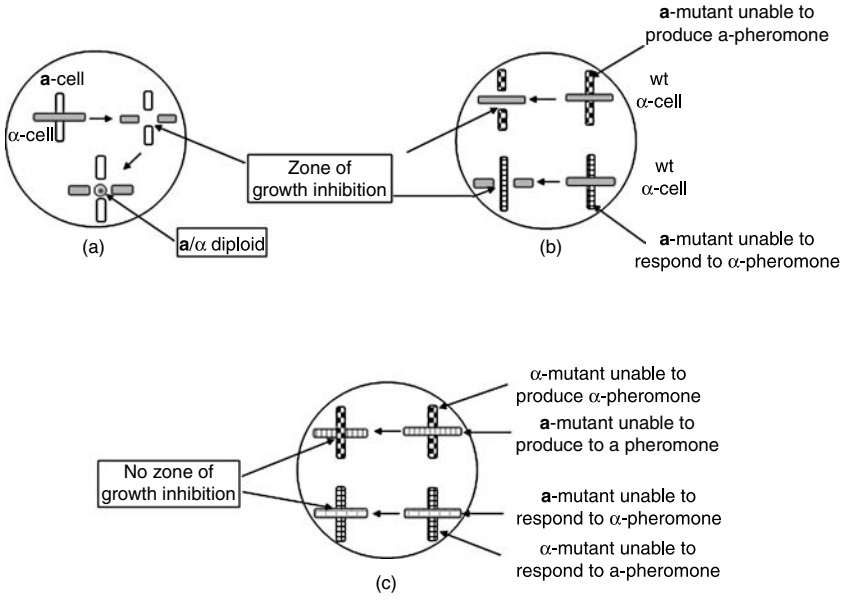

Figure 6.7 Scheme to isolate mutants defective in pheromone signaling by observing growth inhibition of cells in the presence of pheromone. (a) Wild-type yeast cells of opposite mating type show a zone of growth inhibition where the streaks of growing cells cross each other. The diploid cells produced as a result of mating appear as new growth because they are resistant to pheromones. (b) A mutant showing growth inhibition with no effect on the wild type indicates a defect in the production of the pheromone (top), whereas appearance of a zone of growth inhibition around wild type indicates a mutant that does not respond to the pheromone (bottom). (c) Absence of a zone of growth inhibition in the presence of mutant ***a*** and α-cells.

Briefly, streaking yeast cells of opposite mating type on an agar plate in the form of a cross (cross-streaking) results in the appearance of a "zone of growth inhibition" in both the strains as a result of pheromone-induced growth arrest. The "zone of growth inhibition" is eventually populated by the growth of diploid cells, arising as a result of mating and which are non-responsive to pheromones (Figure 6.7a). However, if mutant **a**-cells defective in producing **a**-pheromone are cross-streaked on a plate with wild-type a-cell, a zone of growth inhibition is observed around the mutant only because a-pheromone produced by a-cells arrests the growth of **a**-cells (Figure 6.7b, top). Conversely, if mutant a-cells defective in responding to **a**-pheromone is plated with wild-type **a**-cells, a zone of growth inhibition is observed only around the a-cells (Figure 6.7b, bottom). Finally, if both mutant **a** and a-cells are plated together, no zone of growth inhibition is observed (Figure 6.7c).

Using such a simple visual selection procedure, mutants defective in sending or responding to pheromone signals are identified and the genes cloned by functional complementation. The pheromone pathway has been extensively analyzed by biochemical and molecular genetics techniques, providing us with a wealth of knowledge of a eukaryotic signal transduction pathway (Dohlman and Thorner, 2001).

The biochemical cascade in the mating pathway of *S. cerevisiae* involves coordinated function of many proteins that work harmoniously, akin to a symphony orchestra. The multi-protein signaling complex is schematically shown in Figure 6.8. The receptors are proteins with seven transmembrane segments embedded in the plasma membrane. These receptors are named G-protein-coupled receptors (GPCRs) because they are associated at

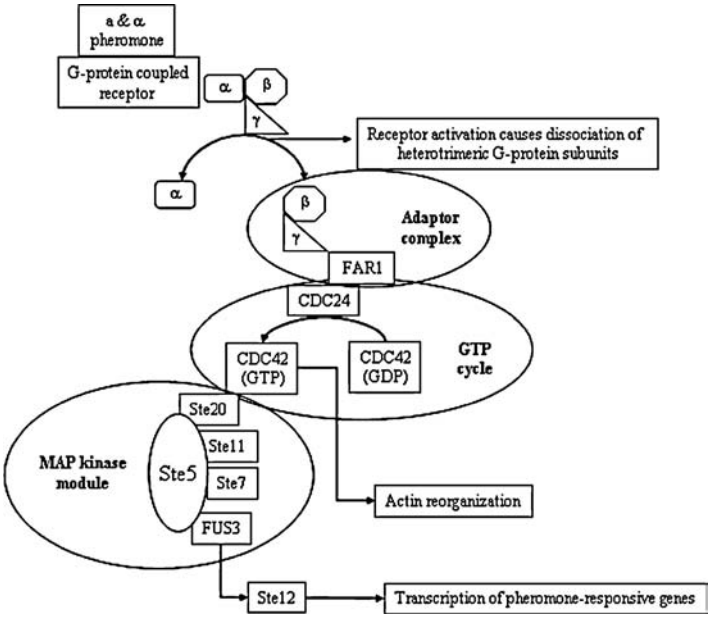

Figure 6.8 Mechanism of pheromone signaling in *S. cerevisiae*. Binding of pheromone to the G-protein coupled pheromone receptor induces expression of pheromone responsive genes by downstream kinases.

the cytoplasmic side to a heterotrimeric protein complex, the G-proteins. The three protein subunits forming the trimeric complex are named α, β and γ. The pheromone receptors are activated by pheromone binding at the extracellular face, which induces a conformational change in the receptor resulting in the dissociation of the α subunit from the β–γ heterodimer. The β–γ complex functions as an adaptor protein to recruit other signaling molecules. Pheromone signaling activates two separate signaling complexes. The first complex, immediately downstream from the adaptor complex, activates *CDC42p* by exchanging GDP with GTP, catalyzed by *CDC24p* (GTP cycle). Active *CDC42p* regulates two separate cellular processes: reorganization of the actin cytoskeleton leading to the appearance of mating projections and activation of the MAP kinase module. The kinase module contains four separate kinases brought in close proximity to each other by binding to the adaptor protein *Ste5*. A cascade of protein phosphorylation by kinases leads finally to the activation of *Ste12* transcription factor. *Ste12* translocates into the nucleus and induces transcription of pheromone-responsive genes. These genes regulate mating and nuclear fusion to begin the diploid phase of yeast life cycle. The molecular mechanisms governing pheromone signaling reflects in composition and characteristics the fundamental nature of signaling pathways found in all higher organisms.

6.5 PROTEIN TARGETING

Proteins coded by the nuclear genome are synthesized in the cytoplasm and must be delivered to different membrane-bound organelles within the cell or secreted outside. How does the cell accomplish this? *S. cerevisiae* played a leading role in identifying the major players in this cellular choreography and early conceptual breakthroughs that came from mutant hunts and genetic analysis.

To identify the genes which, when mutated, would cause a defect in protein secretion, Peter Novick and Randy Schekman used two marker proteins, phosphatase and invertase, whose secretion is easily detected by simple colorimetric assays (Novick and Schekman, 1979; Novick et al., 1980). Yeast cells were mutagenized and plated. Colonies were selected that were defective in the secretion of both enzymes at the restrictive temperature 37°C but not at permissive temperatures of 22 to 24°C. The mutants obtained were organized into 23-complementation groups, suggesting participation of at least 23 genes involved in the secretory pathway. The *ts* mutants are tools to examine the function of essential genes; in addition, they facilitate capturing and analyzing intermediate steps in complex biological processes by imposing the defect at the restrictive temperature. For example, at the restrictive temperature, *SEC18* mutant accumulates 50-nm vesicles containing proteins with pattern of glycosylation specific to the endoplasmic reticulum. This suggests that the *SEC18* gene product functions in the fusion of the ER-derived vesicles with the Golgi-membrane. Many of the proteins involved in vesicular transport identified by biochemical studies of mammalian cell-free systems have been confirmed by genetic approaches using yeast.

The mutants were analyzed to define the order of events in the secretory pathway by the method of double mutant analysis first employed by L. H. Hartwell in 1974 to describe the sequence of events in the yeast cell cycle. Briefly, electron microscopic analysis of the mutants belonging to each of the 23-complementation groups indicated that the mutants specifically accumulated three different membrane-enclosed structures when shifted to the non-permissive temperature. They were either endoplasmic reticulum

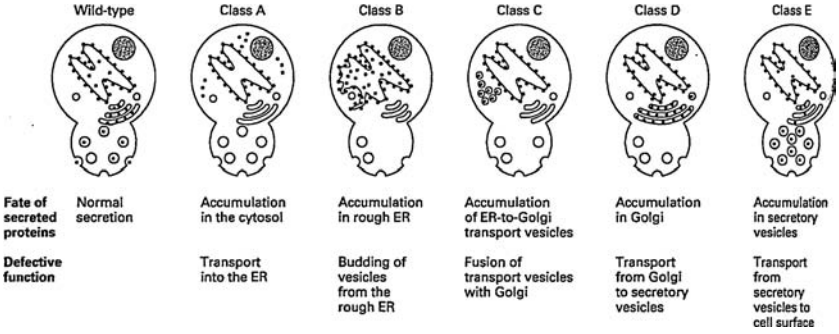

Figure 6.9 Use of yeast mutants in identification of steps in protein secretion. (From Lodish, H. et al., *Molecular Cell Biology*, © 1995, Scientific American Books, Inc. With permission from W.H. Freeman Company.)

structures or cup-shaped structures called "Berkeley bodies" or 80 to 100 nm vesicles. Very rarely a single mutant showed over-representation of more than one structure, suggesting that each mutant is blocked at a discrete step in the process. In double mutant analysis, two mutants that accumulate different structures are combined and its organellar structure is analyzed. It is expected that a double mutant would accumulate a structure that corresponds to the earliest block. Using this method, the secretory mutants (*sec*-mutants) were placed along a linear pathway reflecting the major steps in the secretory process (Figure 6.9).

6.6 MITOCHONDRIAL BIOGENESIS

Mitochondria are energy generating organelles of eukaryotic cells believed to have originated from a symbiotic association between an oxidative bacterium and a glycolytic proto-eukaryotic cell. The endosymbiotic origin of mitochondria is reflected in its bilayered membrane structure, about 86 kb circular genome, organelle-specific transcription and translation and protein assembly systems (Tzagoloff and Myers, 1986). However, during the stabilization of the symbiotic association, the majority of the mitochondrial genes were transferred to the nuclear genome. Recent analysis has revealed that around 477 proteins are required for mitochondrial function in yeast of which only 17 are coded by the mitochondrial genome; the remaining are nuclear encoded. This partial genetic autonomy of the mitochondria is borne out from the analysis of yeast *mit*$^-$ mutants that had point mutations or small deletions in the mtDNA. A second class of mutants was isolated that had a petite phenotype (pet mutants forming small colonies) when grown on a non-fermentable source of carbon such as glycerol, ethanol and lactate. Genetic analysis of these mutants revealed that unlike *mit*$^-$ mutants, *pet* genes were nuclear. Hundreds of *pet* genes were identified by exhaustive genetic screens and shown to regulate mitochondrial transcription, translation and assembly of the electron transport chain. More recently, yeast deletion mutants were used for the identification of new *pet* genes (Steinmetz et al., 2002). Briefly, 4706 homozygous deletion mutants were grown in nine different growth media and their growth characteristics in non-fermentable carbon sources analyzed to identify

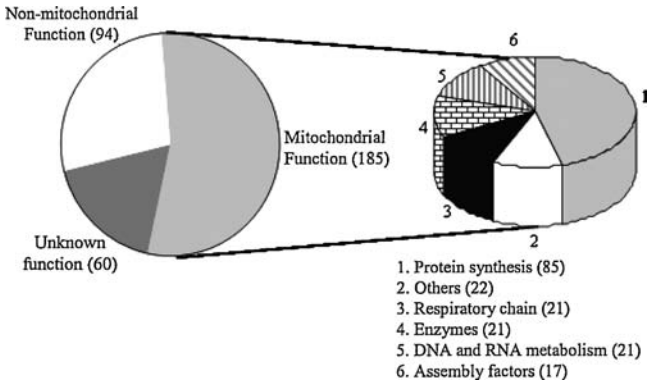

Figure 6.10 Participation of *pet* genes in biological processes. Genes were annotated using Gene Ontology (GO) resources (http://www.geneontology.org).

pet mutants. Altogether, 341 *pet* genes belonging to different classes were identified. The function of 185 mitochondria-specific genes is shown in Figure 6.10. About half of the genes participate in protein synthesis, which is reasonable considering the fact that about 95% of mitochondrial proteins are coded by the nuclear genome.

That both nuclear and mitochondrial genes function together in mitochondrial biogenesis came from the work of Schatz's group (Schatz, 2001). They demonstrated that when yeast cells are grown under anaerobic conditions, their mitochondria are devoid of cytochromes and several other proteins and are difficult to detect by electron microscopy. These structures called proto-mitochondria are converted into functional mitochondria when cells are shifted to aerobic condition. This reversible process became a useful system to demonstrate that the assembly of the electron transport chain on the mitochondrial inner-membrane required the participation of both nuclear and mitochondrial genes. Especially noteworthy was the finding that in the absence of mitochondrial protein translation, cytochrome-c1 encoded by the nucleus became highly susceptible to proteolysis (Ross and Schatz, 1976). The increased susceptibility was not due to increased synthesis of proteases by the petite mutant but due to improper assembly and incorporation of cytochrome-c1 on the mitochondrial membrane. This finding led to the discovery and characterization of an elaborate system by which proteins coded by the nuclear genes finally reach the mitochondrial matrix after traversing the double bilayered membrane of the organelle.

In the early 1990s, a breakthrough in the area of mitochondrial protein import was made by research groups of Gottfried Schatz and Walter Neupert, who established methods for the isolation of mitochondria from yeast cells and set up *in vitro* mitochondrial translocation assays using labeled proteins (Sollner et al., 1989). Briefly, mitochondria from yeast cells are mixed with proteins labeled with ^{35}S-methionine by *in vitro* transcription-translation in rabbit reticulocyte lysates. Following incubation, mitochondria are isolated by density gradient centrifugation and the fate of the labeled proteins analyzed by biochemical and microscopic techniques. A common method involves the treatment of the incubation mixture with proteases and examination of the fate of the labeled proteins by gel electrophoresis. Complete translocation of the protein into the mitochondria renders them resistant to proteolytic digestion. Three significant conclusions were reached from these early studies. First, the mitochondrial outer membrane

bears the import receptors that bind proteins destined to the mitochondria. Second, the proteins destined to the mitochondria carry import signals that are recognized by the import receptors. Third, mitochondrial protein import is an energy-driven process requiring ATP and a potential gradient across the mitochondrial membranes. In the last decade, the biochemical components of this complex process have been identified. Two specific receptor complexes have been characterized that reside on each of the two membranes. The "Tom" complex (translocase of outer membrane) and the "Tim" complex (translocase of inner membrane) form the translocation pore through which proteins are translocated from cytosol into mitochondria. About 30 proteins are present in these two complexes. Proteins destined to the mitochondria are unfolded before they can be recognized by the components of the "Tom" complex. The heat shock protein *hsp70* and chaperone protein MSF mediate the process of unfolding and the stabilization of the unfolded protein by using the energy of ATP hydrolysis. Once the protein traverses the outer membrane, the "Tim" complex threads it in. Proteins residing in the mitochondrial lumen carry a mitochondrial import signal at their N-terminus that is processed by luminal peptidases (Wiedemann, et al. 2004). A simplified view of the mitochondrial protein import process is shown in Figure 6.11.

Like the eukaryotic cell cycle, mitochondrial protein import too is a conserved biochemical event. This concept is supported by functional complementation of yeast mitochondrial import mutants by mammalian genes and also from sequence similarities between yeast and mammalian mitochondrial import proteins. Significantly, function of several human disease genes has been revealed by the function of their yeast homologs (Steinmetz et al., 2002). As an example, when the gene associated with human deafness dystonia syndrome was cloned by positional cloning, no function could be assigned to the DDP1 peptide except that it had an N-terminal mitochondrial localization signal. Soon, however, a family of proteins from yeast bearing striking homology to DDP1 was characterized as components of the "Tim" complex. The human protein has been shown to function as a part of the yeast protein import machinery (Foury and Kucej, 2002). So far, about 102 human diseases have been attributed to defects in mitochondrial function. The yeast system offers a great tool to analyze the function of these human genes (Koutnikova et al., 1997).

6.7 FUNCTIONAL GENOMICS

In 1996, 92 collaborating laboratories in the USA, Canada, the UK and Japan published the complete sequence of the *S. cerevisiae* genome (Goffeau et al., 1996). This opened up research on how genetic instructions specify a eukaryotic cell, heralding the era of comparative genomics—comparing sequences between different species—and to learn about the basic mechanisms of life that could lead to understanding how genes cause diseases and of finding rationales for treatment. Some examples of spin-offs from the yeast genome sequence are given below.

The 12 million bases in the yeast genome predicted 6213 protein-encoding genes, of which 3470 genes (56%) have homologues in other organisms. The remaining 2743 genes lack identifiable homologues in any organism. When these genes were compared with genes from 13 species of yeast, 1712 genes showed homologues, indicating their common function in this group of organisms. Among the conserved genes are those involved in cell wall biosynthesis and the pheromone response. About 78% of the genes are assigned to biological

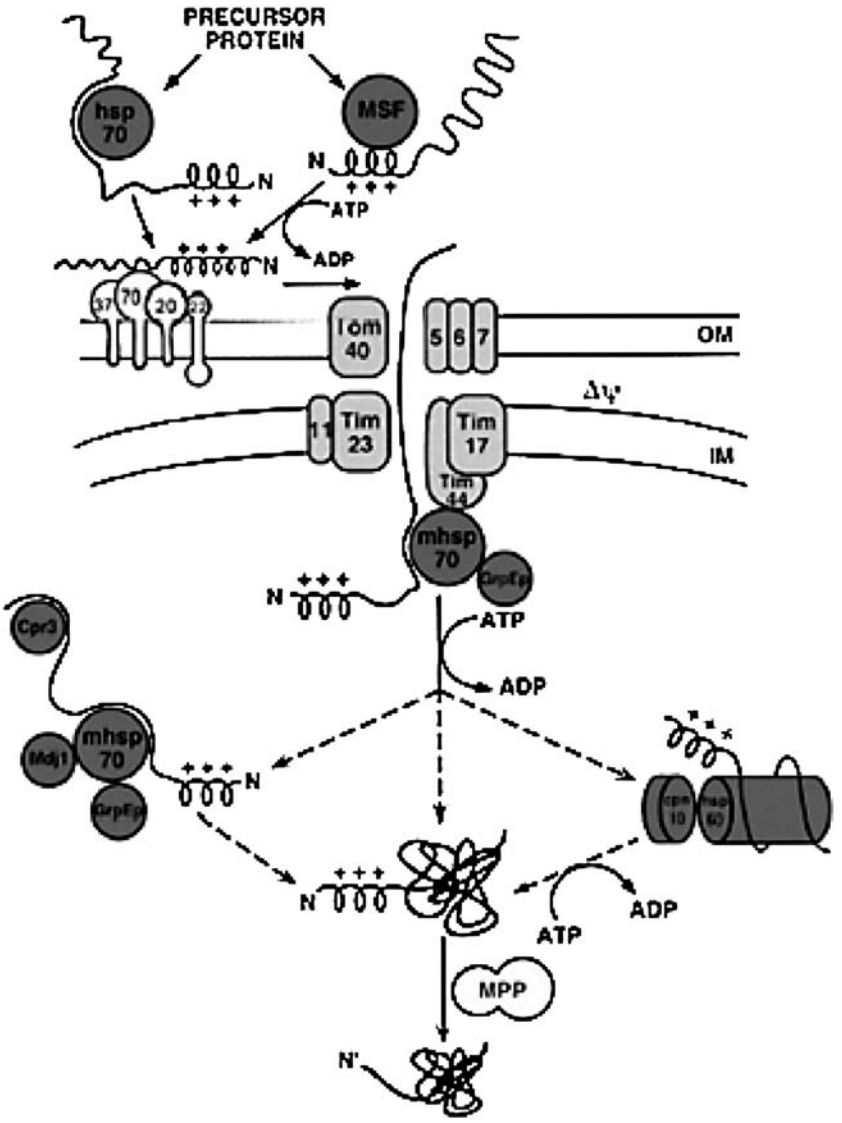

Figure 6.11 Components of the mitochondrial protein import machinery. Precursor proteins undergo unfolding in the presence of heat shock protein-70 and mitochondrial import stimulating factor (MSF) proteins. The mitochondrial import receptor complex binds the positively charged N-terminal signal sequence before the protein is transferred to the "Tom" complex in the outer membrane (OM). The protein is transferred to the components of the "Tim" complex in the inner membrane (IM), which pulls it into the matrix where it is folded in the presence of chaperone proteins (mitochondrial heat shock protein-70 and Gro-related protein-E). Once folded, matrix-processing peptidase (MPP) cleaves the signal peptide. Reproduced from Figure 1 in Schatz, G. (1996), *J. Biol. Chem.* 271:31763–31766. With permission from American Society for Biochemistry and Molecular Biology.

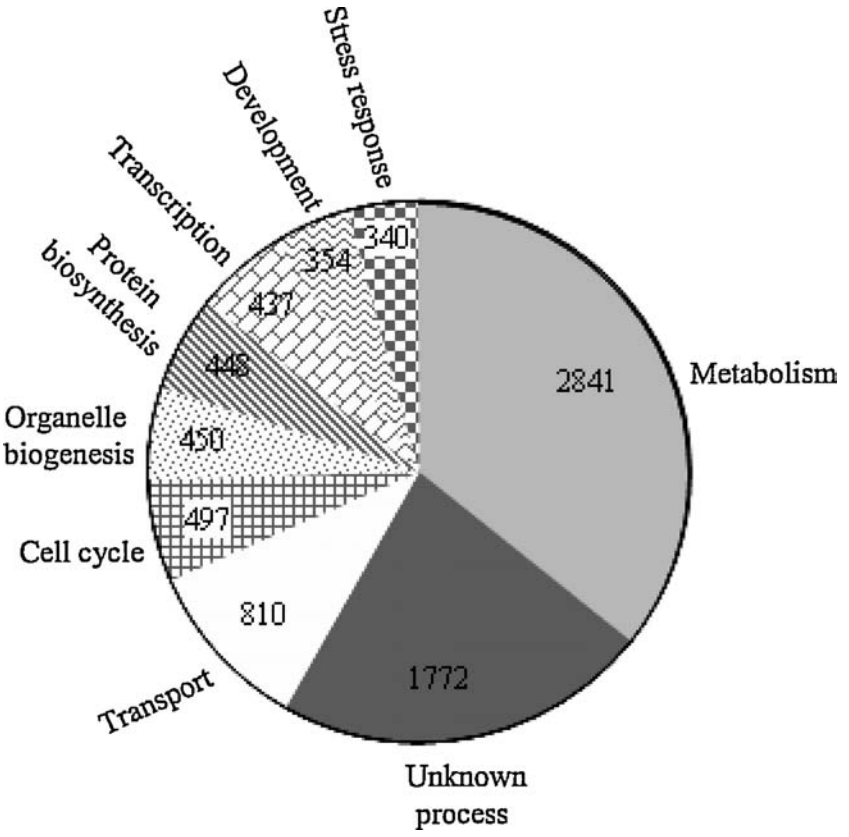

Figure 6.12 Distribution of yeast genes in different biological processes. A single gene may participate in more than one cellular process. Numbers were compiled from Gene Ontology (GO) resources (http://www.geneontology.org).

processes based on sequence homology, genetic and biochemical evidences (Figure 6.12). For the first time, the availability of a complete sequence of an eukaryotic organism accelerated the discovery and use of novel experimental approaches to understand gene function described in the next sections.

6.7.1 Number of Yeast Genes

Until recently, the total number of functional genes in an organism was estimated by saturation mutagenesis. What this means is that any gene that is associated with a function can be identified by the loss of function after mutagenesis. Therefore, if an organism is mutagenized and enough mutants are screened, the total number of mutants can give a rough estimate of the total number of functional genes. The yeast genome sequence revealed about 6200 genes, two times more than that predicted from saturation mutagenesis. A second approach to analyze gene function is by targeted disruption of genes, possible only after the genes have been identified and their sequences determined. R.W. Davis and his colleagues disrupted 5916 genes (96.5% of total) and analyzed the behavior of mutants under a variety

of nutritional and environmental conditions (Glaever et al., 2002). In their experiments, each gene was precisely deleted from the start to the stop codon and replaced with a "deletion cassette" containing a selectable marker kanamycin. The kanamycin gene in each deletion cassette was flanked on either side by two unique 20-nucleotide sequences. These unique sequence tags can be viewed as a barcode, a permanent identifier of each deletion mutant. This clever trick of tagging every deleted gene permitted the researchers to carry out growth experiments with many deletion mutants in parallel. In a typical experiment the relative contribution of genes for growth on galactose was examined with the objective of assigning functions to as of yet uncharacterized genes (Figure 6.13).

Twelve deletion mutants were grown in a medium with galactose as the sole carbon source with the relative proportion of each mutant cell in the mixed culture determined by quantitating the relative amount of each tag present in the culture by microarray technology (described in the next section). Analysis of growth characteristics of each mutant in galactose media revealed the impact of individual genes on the utilization of galactose. By this method, the functions of two novel genes *YML090W* and *YML077W* in

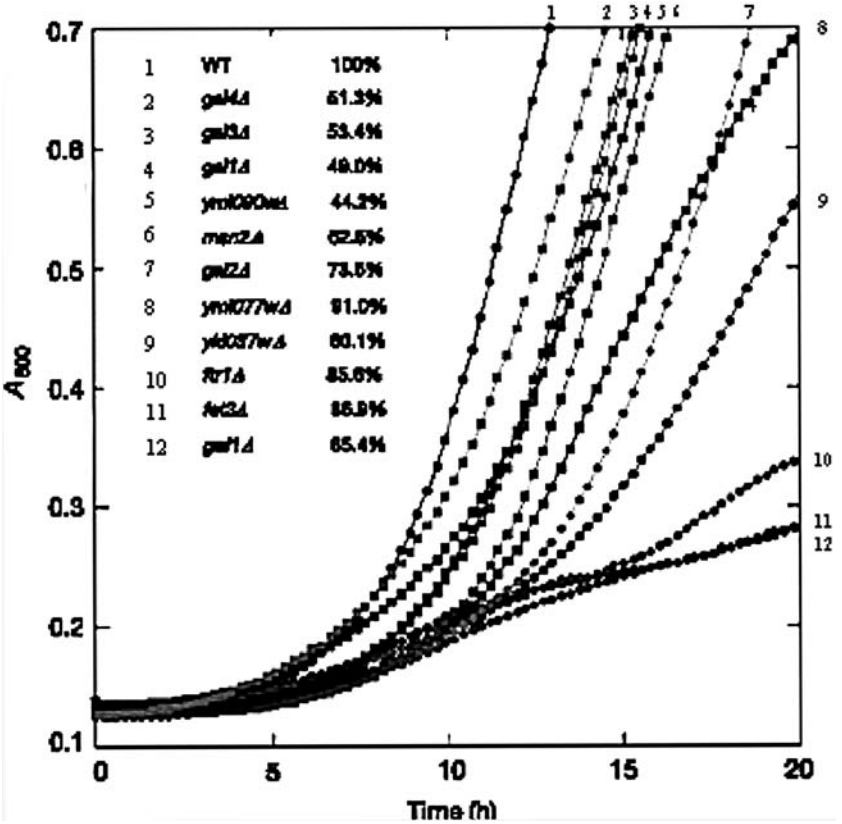

Figure 6.13 Measuring fitness of deletion strains of *S. cerevisiae* in galactose medium. Strains numbered from 1 to 12 were grown together in the same tube. The growth of each strain was quantitated by quantifying the barcodes associated with each mutant using an oligonucleotide array (microarray) as described in the text. (From Glaever et al. (2002).)

the utilization of galactose were revealed. The collection of deletion mutants is being used as a functional genomic strategy for the analysis of complex cellular processes and metabolic pathways.

6.7.2 Expression Pattern of Genes Using DNA Microarrays

Paradigm shift in science always goes hand-in-hand with technological breakthroughs. The program by which a complex body develops from a single fertilized cell requires the understanding of the spatial and temporal regulation of gene transcription in response to external and internal cues. In 1995, Patrick O. Brown and his colleagues at Stanford University introduced the DNA microarray technique for analyzing global expression of genes (Schena et al., 1995). The yeast cell cycle was one of the first biological processes to be interrogated by this new technique. The commercial yeast microarray contains 6200 genes covalently linked on glass slides as about 100 μm dots in grids of 96 or 384 spots. Each gene is amplified by polymerase chain reaction (PCR) and the purified DNA is used for printing the microarrays. Microarray technology (Figure 6.14) is fully automated, carried out by robots to increase throughput and minimize error. The microarray experiment measures the relative abundance of messages in a cell, revealing a picture of a cell's transcriptome. It has been applied successfully to identify induction and repression of genes during specific cellular processes. The first gene expression analysis examined the genes that are regulated in a cell-cycle dependent manner in yeast (Spellman et al., 1998) by comparing the relative abundance of mRNA as cells progressed through the cell cycle. Messenger RNA was harvested at defined time points to capture different phases of the cell cycle and converted into complimentary DNA (cDNA) using appropriate primers and the enzyme reverse transcriptase in the presence of red (Cy5) and green (Cy3) fluorescently labeled nucleotide precursors. Labeled cDNA (Cy3) from an asynchronous culture (control) was mixed with labeled cDNA from the synchronous culture (Cy5) and hybridized to a DNA microarray containing the yeast genes. The cDNA sequences representing individual transcripts hybridized specifically to corresponding gene sequences on the array. The fluorescence associated with each spot was quantitated using a microscope (microarray reader) that illuminates each spot with a laser beam and measures the fluorescence associated with each dye separately to estimate the relative abundance of the transcript by the ratio of the red to green fluorescence. About 800 genes were found to be cell cycle regulated, which could be clustered into groups, whose expression correlated with specific phase of the cell cycle. For example, about 100 genes were found to be co-regulated in G1. Many of these genes function in establishing cellular polarity and initiation of bud growth, providing a molecular link between budding with the position of the cell in G1. The microarray technique has become a standard tool to understand the mechanism of disease incidence and progression and has been applied successfully in cancer.

6.7.3 Mapping Transcription Network

In the early 1960s, bacterial genetics laid the concept of regulatory circuits controlling expression of genes. In its simplest form, circuits are turned "on" or "off" by the binding of transcription factors or repressors to the upstream regulatory sequences of genes respectively. Expression analysis by microarrays described in the earlier section revealed the co-regulated expression and repression of sometimes hundreds of genes during specific cellular

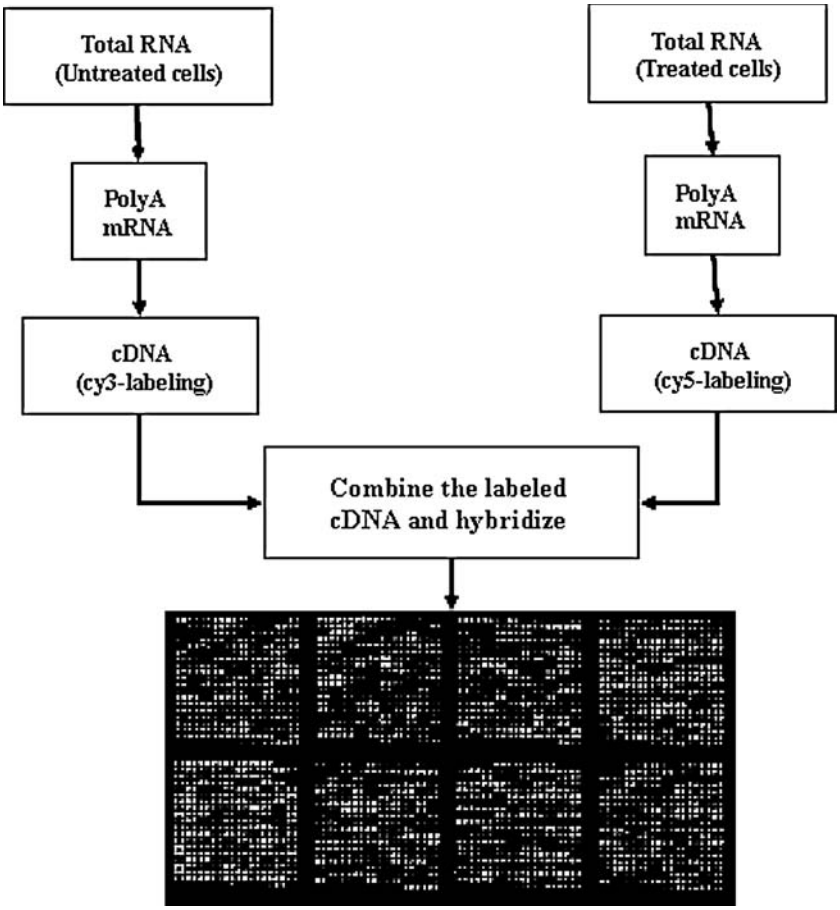

Figure 6.14 A schematic representation of a microarray experiment. Labeled cDNA prepared from untreated and treated cells was mixed and hybridized on a microarray slide spotted with 6200 yeast open reading frames (DNA sequences with initiation and termination codons). The red spots represent higher abundance of cyanin5-labeled genes and green spots represent higher abundance of cyanin3-labeled genes. Yellow spots are genes present in equal abundance (blending of red and green color in equal proportion). (See color insert following page 140.)

events. Richard A. Young used the microarray technique to identify regulatory sequences bound by all transcription factors encoded by the yeast genome (Lee et al., 2002). Based on the expectation that the promoter elements are located within the non-coding regions upstream of every gene, the group amplified the non-coding sequences between genes (intergenic regions) and spotted them on slides to create microarrays of all promoter regions of yeast. Next, they identified 141 genes in the yeast genome that are predicted to code for transcription factors and tagged them with an epitope (myc-tag) at the c-terminus. Each epitope-tagged transcription factor was inserted into its native genomic locus by homologous recombination and analyzed for the level of expression by Western blot using an antibody specific to the myc-tag. To identify the DNA-binding sequences of the transcription

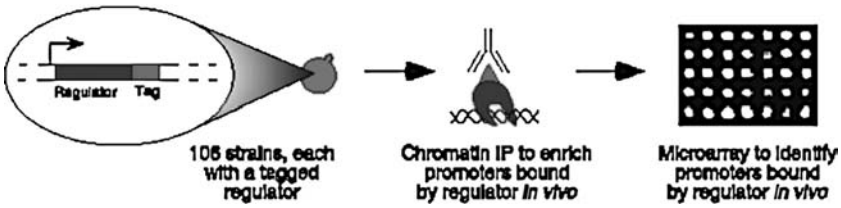
Regulator
Tag
106 strains, each with a tagged regulator
Chromatin IP to enrich promoters bound by regulator *in vivo*
Microarray to identify promoters bound by regulator *in vivo*

Figure 6.15 Genome-wide location analyses of yeast transcription regulators. Tagged transcription regulators are expressed in yeast. Bound regulators are crosslinked to DNA and DNA-protein complex is digested with DNase to obtain fragments of DNA complexed with regulators. Bound regulators are immunoprecipitated using antibodies specific to the tag. The DNA associated with the regulator protein is isolated and hybridized to microarrays to identify the sequence. The binding of the transcription regulator is mapped to the sequence. (Lee et al. (2002), Transcriptional regulatory networks in *S. cerevisiae, Science* 298, Figure 1, AAAS. With permission.) (See color insert following page 140.)

factors, a genome-wide location analysis called ChIP (crosslinking chromatin immunoprecipitation) was performed (Ren et al., 2000). The strategy is shown in Figure 6.15. Each yeast strain expressing a unique myc-tagged transcription factor was grown in rich medium and the DNA cross-linked to bound proteins *in vivo*. The protein-DNA complex was digested with the enzyme DNAse to cleave DNA into smaller fragments. The transcription factor-bound DNA fragments were enriched by immunoprecipitation using an antibody against the tag. After isolation of bound DNA from the protein-DNA complex, it was labeled and hybridized to a microarray slide spotted with the intergenic DNA. A positive hybridization signal identified specific intergenic regions to which the transcription factor was bound. Sequence comparison among the intergenic regions that lighted up with each transcription factor identified conserved motifs recognized by the transcription factor. A genome-wide search for the presence of motifs in upstream sequences of genes resulted in the discovery of genes that are co-regulated under the control of the transcription factor. Using this approach it was revealed that about 200 genes are regulated by SCB binding factor (SBF) and MluI cell cycle box (MCB) binding factor (MBF) transcription factors during G1-S phase transition of the cell cycle (Iyer et al., 2001; Simon et al., 2001).

6.8 PROTEOMICS AND SYSTEM BIOLOGY MODELING

A cell continuously senses environmental stimuli and relays that information inside across membranes to trigger a response. The process of signal transduction occurs by sequential transfer of information via protein-protein interaction. Take the example of a yeast cell growing in a glucose-rich environment suddenly encountering galactose. It senses the change in composition of the carbon source, shuts off the expression of genes required for glucose metabolism and turns on the genes required for galactose utilization. What is the mechanism by which the cell makes necessary adjustments? It is known that galactose itself can induce changes in gene expression within the yeast cells. The mechanism of regulation of gene expression by galactose is mediated by altering the interaction between *GAL4p* and *GAL80p*. As shown in Figure 6.16, in the absence of galactose *GAL4p* is in complex with *GAL80p* and is functionally inactive as a transcription factor. However, the presence of galactose induces recruitment of *GAL3p* to *GAL4-GAL80* complex. This

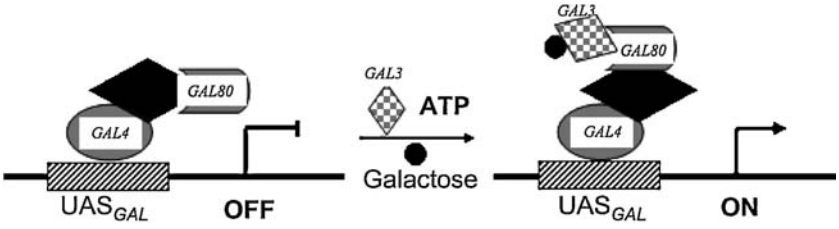

Figure 6.16 Transcriptional regulation of galactose-inducible genes is mediated by protein-protein interaction. In the absence of galactose, *GAL4* transcription factor is kept transcriptionally silent as a result of interaction with the negative regulator *GAL80* protein. This inhibition is relieved in the presence of galactose by *GAL3* protein, which binds *GAL80* and displaces it from the *GAL4* transcription activation domain.

binding revives the transcriptional competence of *GAL4p* by relieving the inhibition caused by *GAL80p*. As a consequence, *GAL4* induces transcription of genes required for galactose metabolism.

Identifying the interacting partners of every protein in a cell is paramount to the understanding of how cells initiate and coordinate myriad functions to maintain homeostasis. Stanley Fields devised a yeast two-hybrid method of discovering protein-protein interaction (Fields and Song, 1989). Briefly, the two-hybrid method detects protein-protein interaction by transcriptional induction of reporter genes. Two separate reporter systems are used, a nutritional marker that allows growth of yeast strains on a selective medium and an enzyme (beta-galactosidase), whose activity can be measured by colorimetric assay. The transcriptional induction of the reporter genes is dependent on the availability of a functional *GAL4* protein. *GAL4p* is a modular transcription factor with a distinct DNA-binding and transcription activation domain. Each domain retains its function in the absence of the other. In the two-hybrid method, the DNA-encoding the test proteins are fused *in vivo* either to the DNA-binding domain or to the activation domain of the *GAL4* transcription factor by homologous recombination in yeast. By themselves, the fusion proteins are incapable of inducing transcription. However, if the two test proteins interact with each other, the two domains of the transcription factor will be brought close together to reconstitute a functional *GAL4* protein. As a result, the reporter gene under the control of *GAL4* promoter will be transcriptionally induced. Positive interactors are selected by monitoring the expression of beta-galactosidase using a synthetic substrate that turns blue following the action of beta-galactosidase. Yeast cells containing positive interactors grow into colonies that appear bluish when grown on selective plates. The two-hybrid method is shown in Figure 6.17. Yeast two-hybrid libraries are constructed by fusing cDNAs (obtained from mRNA) with the activation domain of *GAL4* protein (AD-fusion) and cloned in the prey plasmid. The genes of interest are fused to the DNA binding domain of *GAL4* protein (BD-fusion) and cloned in a bait plasmid.

The two-hybrid technology was employed for the first time to initiate a genome wide interaction screen in *S. cerevisiae*. The complete sequence information of the yeast genome permitted cloning of all the roughly 6200 open reading frames (ORFs). Each gene (ORF) was screened against a yeast two-hybrid library and their interactors were identified. Since the first high throughput screen (Uetz et al., 2001) that detected around 1000 interactions, the yeast interaction database today holds about 5000 unique interactions. A

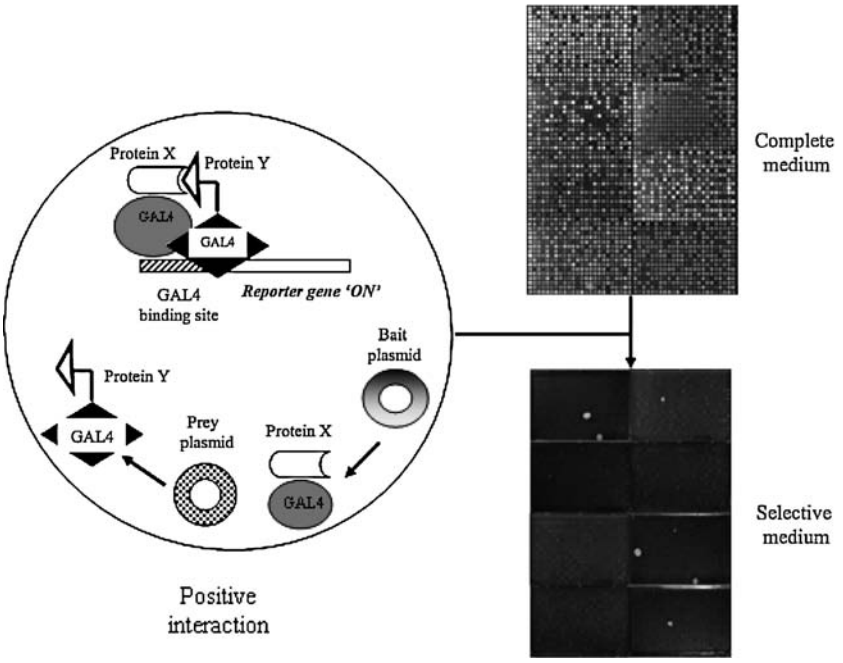

Figure 6.17 Yeast two-hybrid system. Yeast cells are transformed with plasmids expressing two separate fusion proteins, one containing the *GAL4* DNA-binding domain (bait plasmid) and the other with the *GAL4* activation domain (prey plasmid). The DNA-binding and the activation domains are brought closer to each other into a stable complex if the fusion proteins interact with each other, turning on the expression of the reporter gene. Positive interactors show growth on selective plates.

recent analysis of the interactions has revealed many biologically relevant protein complexes associated with distinct cellular processes (Bader et al., 2004).

A second approach of studying protein-protein interaction is by mass spectrometry. The major impetus of using a yeast system to analyze protein complexes by this method was the fact that the data generated using this developing technology can be cross-validated easily with the vast amount of genetic, biochemical and molecular biology information already available in this model organism. Two drug discovery companies have tagged about 1900 yeast proteins with an epitope tag and expressed them in yeast under the control of their native promoter. Protein complexes were purified from yeast lysates by affinity chromatography using an antibody against the tag. After separating individual proteins in the complex by denaturing gel electrophoresis, each protein band was excised from the gel, digested with trypsin and identified by Matrix Assisted Laser Desorption Ionization-Mass Spectrometry (MALDI-MS). Together, the two studies identified 3018 interacting proteins (about half of all yeast proteins) distributed in a variety of biologically relevant complexes (Ho et al., 2002; Gavin et al., 2002).

High throughput protein-protein interaction analysis generates huge amount of data that is not easy to tease apart to extract biologically meaningful interactions from the noise. A straightforward approach to assess the quality of any interaction is to determine

whether the interacting proteins are expressed together at any given time and also whether they localize within the same cellular compartment. *S. cerevisiae* was used to examine the expression and localization of the yeast proteome.

Erin K. O'Shea and Jonathan Weissman carried out a global analysis of protein localization in yeast (Ghaemmaghami et al., 2003; Huh et al., 2003). The objective of this experiment was two-fold: first, to localize proteins into specific cellular compartment, using a GFP tag; and second, to determine quantitatively the steady state level of all proteins in a yeast cell by western blot using a myc-tag. The researchers therefore generated two different strains of yeast in which 6109 of the 6243 predicted yeast-ORFs were fused either with a myc-tag or with a GFP-tag, providing for the first time a comprehensive view of the expressed proteome and its subcellular localization in a eukaryotic cell. Expression of proteins by western blot and protein fluorescence in yeast cells under normal growth conditions revealed that around 80% of the proteome is expressed. The abundance of individual proteins ranged from 50 to 1,000,000 molecules under normal growth conditions. The examination of protein localization at high resolution and sensitivity was achieved by GFP fluorescence. The study was able to localize (two-thirds) of the previously unlocalized proteins into 12 subcellular categories. A future goal is to understand how localization of proteins within a cell changes as a result of cellular signaling.

6.9 CONCLUDING REMARKS

For more than half a century yeast has contributed to our understanding of biological processes such as metabolism and enzyme regulation, cell recognition, structure of chromosomes, mechanisms in meiotic recombination, epigenetic effects through mating type switching, cell cycle, the compartmental character of eukaryotic cells, protein targeting, and so on. An area where yeast will provide valuable information is the emerging area of system biology. System biology aims to understand complex cellular behavior such as how cells perceive and analyze signals in a continuously changing environment, or how cell division is linked to the generation of polarity by overlaying information obtained using genomics and proteomics methods with genetic and biochemical techniques. We conclude this chapter with an observation of Davis (2003):

> By a process of natural selection, as it were, yeast has attracted many post-1970 investigators with strong training in all three of vital disciplines: genetics, biochemistry, and molecular biology. Their ability to integrate these disciplines in their research and in their training of newcomers to yeast has led to connectivity of the many levels of organization that defines a model organism. This ability has also led to the explosive growth of the yeast community, which has been so self-sufficient in defining life's fundamental attributes that its members are no longer obliged to read the literature on other fungi. A serious asymmetry prevails in this matter: *Neurospora* and *Aspergillus* investigators ignore the yeast literature at their peril.

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Chapter 7

Aspergillus nidulans: A Model for Study of Form and Asexual Reproduction

> As life processes became domesticated in laboratories, a need to optimize observations led to choices of more suitable material. … But the choices and changes of organism gradually improved our sense of the workings of living things, usually focusing on one attribute at a time.
>
> *Rowland H. Davis*

Many fungi produce conidia on special hyphal branches called *conidiophores*. These are elaborate structures as the inflorescences of higher plants—for example, like the branching of a bunch of grapes, a spike of wheat or a capitulum of dandelion. The conidiophore is a morphological device for the rapid production in a small space of a large number of asexual (mitotically derived) reproductive spores that can be liberated and disseminated by air current, the splash of rain or insects. Conidiophores in different species are of different morphology and this is an important criterion in their identification. Because of the rapid production of large numbers of loose conidia that can be disseminated, fungi are notorious in causing epidemic in plants, spoilage of food and contamination in the laboratory unless precautions are taken.

Aspergillus nidulans (Ascomycotina) is an example of a fungus that illustrates the basic strategy of asexual reproduction. Its conidiophore is a multicellular structure comprised of a relatively small number of cell types, developing in an orderly manner and in a precisely timed sequence. Each of its conidia has a single nucleus, providing a convenient source of identical haploid cells that can be mutagenized for the production of developmental mutants. The phenotype of a colony derived from a single spore is dependent only on the genotype of the nucleus. A collection of different mutants with altered conidiophore morphology can be arranged to determine the time-line of sequence in the differentiation of a multicellular structure. Moreover, the fungus is homothallic, meaning that there is no mating type barrier for sexual reproduction; crosses can be made between any two parents. Because of these features, Timberlake and colleagues chose this fungus and developed transformation protocols, gene replacement and gene disruption techniques for understanding how a multicellular structure is formed for performing a specific function.

7.1 CONIDIOPHORE MORPHOGENESIS

The development of an *Aspergillus* conidiophore can be divided into steps that occur in sequence. It begins with the differentiation of a hyphal cell into a thick-walled foot cell that extends into an aerial stalk of approximately 100 μm (Figure 7.1) and allows the asexual reproductive cells to be pushed out of substratum for dissemination. Second, the stalk swells at the tip to form a globose, multinucleate vesicle which has a diameter of about 10 μm. Third, the vesicle buds out about 60 cigar-shaped uninucleate cells called *metulae*. Fourth, each metula in turn buds out two phialide cells. Fifth, a single nucleus enters into the phialide and by successive mitotic divisions each phialide buds out a vertical chain of conidia with the oldest conidium at the top of the chain. Each conidium is approximately 3 μm in diameter. By this form of development, each conidiophore can produce over 1000 conidia with economy of space and in a short time. We are beginning to understand how genes specify the development of a conidiophore stalk of a definite height that swells into a vesicle, from which are formed a number of metulae- and phialides-precise structures budding a number of spores. The principles elucidated from this relatively simple system apply to embryonic development—how does the embryo mark and measure space and time so that organs and tissues develop on schedule and in the correct locations?

The time line of conidiophore development in *A. nidulans* is as follows (Boylan et al., 1987): Undifferentiated hyphae (0 h) $\rightarrow$ aerial stalk (5 h) $\rightarrow$ vesicle (10 h) $\rightarrow$ metula and phialide (15 h) $\rightarrow$ immature conidia (20 h) $\rightarrow$ mature dark green conidia (25 h).

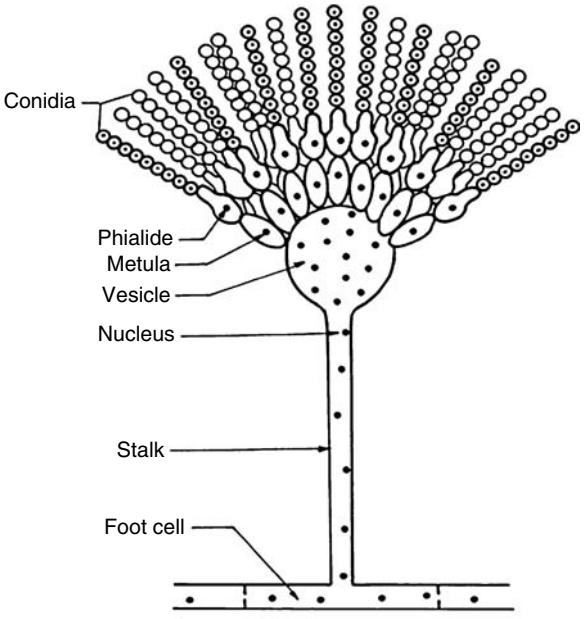

Figure 7.1 Diagrammatic structure of a conidiophore of *Aspergillus nidulans*. Redrawn from Moore (1998).

7.1.1 Developmental Competence

A. nidulans does not conidiate in submerged liquid cultures. However, if the mycelia from the submerged cultures are exposed to air and light for at least 15 to 30 min, conidiophore differentiation takes place. The action spectrum of conidiation showed that light of 680 nm is maximally effective, i.e., the specific wavelength that elicits conidiation is red light. The chemical nature of the photoreceptor in *A. nidulans* is not yet known. Interestingly, the red-light effect is inhibited by far-red light—a property well-known for the phytochrome-mediated responses in the green plants. The *veA1* mutants conidiate both in light and dark conditions (Mooney and Yager, 1990), suggesting that the light requirement is dependent upon the *veA* gene.

By exposing liquid-grown mycelia to air and light at different time intervals, it was determined vegetative cells become competent to form conidiophores after 18 h of submerged growth. It was therefore hypothesized that a defined amount of vegetative mycelium must be formed and developmental competence acquired before mycelium can produce a conidiophore. The nature of competence remains unknown. Mutant strains have been isolated in which the length of growth period before conidiation is altered, suggesting that the switch from vegetative growth to conidiophore development is genetically determined (Axelrod et al., 1973).

7.2 MICROCYCLE CONIDIATION

Although typically some amount of mycelial growth precedes the development of conidiophore, in a type of development called microcycle conidiation, a germinating conidium totally bypasses the mycelium phase and directly develops a conidiophore (Maheshwari, 1991). For example, conidia of *Neurospora crassa* germinate on a nutrient agar medium. Normally the germ tubes fuse and mycelium is formed (Figure 7.2a), from which two types of conidia are produced: blastoconidia, by a process of budding (Figure 7.2b); and arthroconidia, by a process of septation (Figure 7.2c). In some strains grown in submerged-shake cultures, the conidia directly develop conidiophores (Figure 7.2d). In *Aspergillus niger*, microcycle conidiation can be induced under certain conditions of nutrition and temperature regime (Figure 7.3). Apparently, in this type of development, the conidiation genes are activated and expressed precociously. Microcycle conidiation could be a common feature of fungi growing in nature, allowing the formation of asexual propagules in the shortest possible time. The phenomenon of microcycle conidiation suggests that a master regulatory gene controls the expression of a large number of conidiation genes.

7.3 CONIDIATION GENES

Mutants of *A. nidulans* were obtained that are normal in hyphal growth and sexual reproduction but are defective in conidiophore development (Clutterbuck, 1969). Since conidia are not formed, the conidiation mutants are detected by the lack of the wild green color and by their inability to be replica plated—a simple technique that allows colonies producing loose conidia in a petri dish to be sampled by pressing a velvet cloth secured over the top of a cylinder and onto the surface of new medium in a petri dish. The procedure transfers each conidiating colony in a way that the pattern of colonies is maintained on the replica plate. The colonies that are missing because of non-conidiation are identified in the master

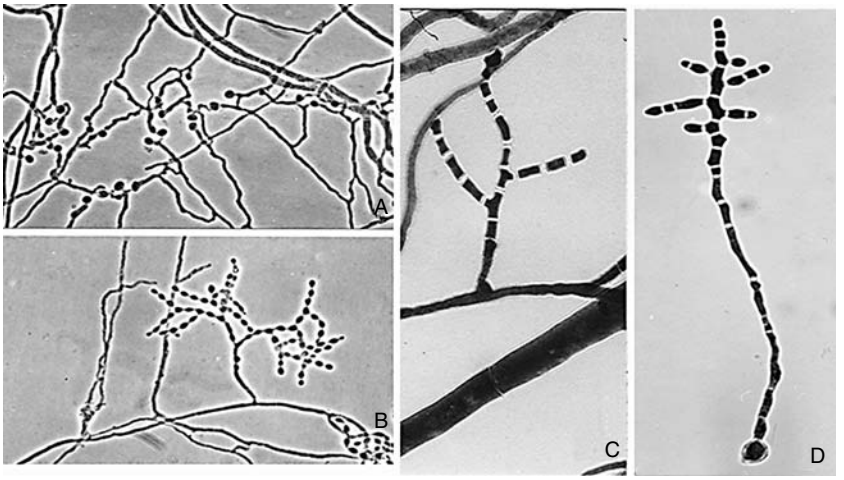

Figure 7.2 Conidiation in a strain of *Neurospora crassa*. (A) Fusion of conidial germ tubes and formation of mycelium. (B) A conidiophore in surface-grown mycelium bearing macroconidia formed by budding. (From Maheshwari (1991), with permission from Society of General Microbiology.) (C) A conidiophore in surface-grown mycelium producing arthroconidia by septation. (D) Microcyclic conidiation in the same strain grown in submerged shake culture.

plate. In one study, the number of conidiation-specific genes was estimated by comparing the frequency of conidiation mutants with the frequency of auxotrophic mutants:

$$\text{number of conidiation genes} = \left(\frac{\text{frequency of conidiation mutants}}{\text{frequency of auxotrophic mutants}}\right) \times \text{number of auxotrophic genes}$$

It was estimated that approximately 100 genes specifically affect conidiation (Martinelli and Clutterbuck, 1971). A larger number of geners (about 1000) was estimated based on the diverse messenger RNA that accumulated specifically during conidiation (Timberlake, 1980). Based on the mutant phenotypes and the comparison of gene expression in wild and conidiation mutants, three genes were identified as essential regulators of conidiophore development: the *bristle* (*brl*) gene controls the swelling of conidiophore stalks into vesicles; the *abacus* (*abaA*) gene controls the budding of conidia by the conidiophore stalk; and the *wet-white* (*wetA*) gene controls the hydrophobicity that is essential for the aerial dissemination of conidia. The *wetA* mutants produce normal conidiophores but the conidia lack hydrophobicity and autolyze, forming droplets at the ends of conidial chains. These three genes are themselves not specific to conidiation—rather, they integrate the expression of other genes that determine the structure of the conidiophore. The genes are therefore considered to be regulatory genes that play a key role in conidiophore development and act in the sequence *brlA* → *abaA* → *wetA*. Using a genomic library, the wild *brlA*, *abaA* and *wetA* genes were isolated by complementation of the mutant strains. The nucleotide sequence of *brlA* showed that it has a short open reading frame (ORF) that regulates transcription. The deduced amino acid sequence of *brlA* protein product can form a secondary structure with a loop of the polypeptide chain, to which a Zn atom can bind.

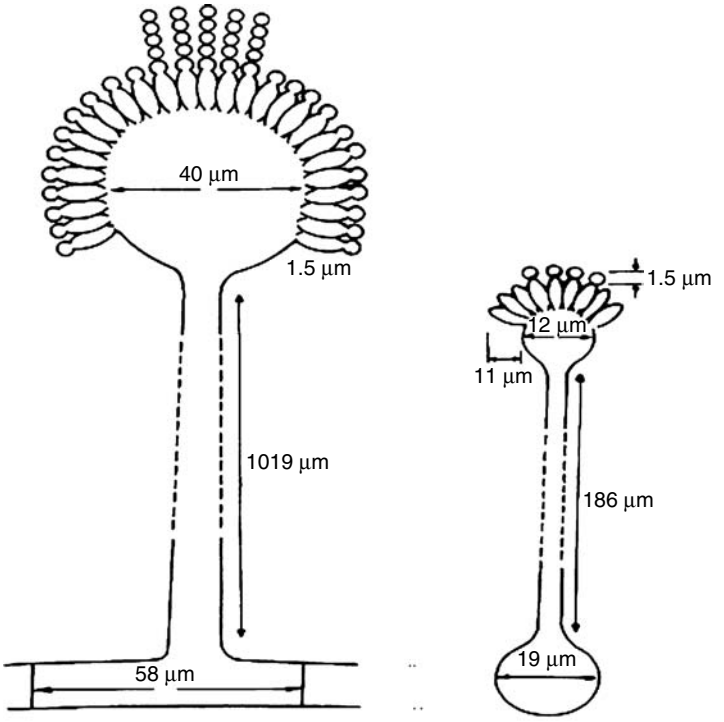

Figure 7.3 Diagram of microcycle conidiation in *Aspergillus niger*. The mycelial phase was bypassed when conidia were subjected to a regime of heat shock. (From Anderson and Smith (1971).)

Since this type of structure is common in DNA-binding proteins, the *brlA* gene is thought to encode a transcription regulator that activates genes required for conidiophore formation (Adams et al., 1990).

To determine whether the conidiation-specific genes occur in a close physical order that reflects their order of action or are distributed randomly in the genome, thirty cDNA clones containing approximately 1.5 kb long DNA inserts were hybridized to poly(A)$^+$ RNA (Orr and Timberlake, 1982). On the basis of the random distribution of conidiation genes, the majority of clones were expected to have only one spore-specific transcript. However, the experimental finding was that many clones hybridized to several spore-specific transcripts of different molecular weights, indicating that the spore-specific genes in *A. nidulans* may be clustered and constitute a functional unit of developmental gene regulation.

7.4 CONIDIATION TRIGGER

Nutrient exhaustion was thought to be the single most powerful general stimulus for the initiation of reproduction in fungi. Adams and Timberlake (1990) questioned whether conidiation development could be induced in the presence of excess nutrients. Adopting the recombinant DNA methodologies, they constructed strains of *A. nidulans* in which the

promoter gene (*p*) from the alcohol dehydrogenase gene (*alcA*) was fused to conidiation regulatory genes (*brlA* or *abaA*) and critically tested the nutrient exhaustion hypothesis of conidiation. The genotype of constructed gene fusion strains is denoted as *alc(p)* :: *brlA abaA* and *alcA(p)* :: *abaA*. The fusion strain germlings were grown as a homogeneous mycelium in submerged cultures containing a carbon source. After three hours, the cultures were shifted to media containing threonine or ethanol to force the induction of the *brlA* or *abaA* gene. The forced expression of the conidiation-specific regulatory genes *brlA* or *abaA* led to a generalized metabolic shutdown as indicated by the reduction in total protein and RNA and to a loss in the ability to take up nutrients from the medium. Vegetative growth ceased and conidia were formed at the ends of hyphae, although without the formation of vesicle, metula and phialide (Adams et al., 1988; Adams and Timberlake, 1990). The ability to induce conidiation by direct activation of the *brlA* gene suggested that *brlA* mediates the development switch from the polarized growth of stalk cell to budding growth leading to conidium formation. It was inferred that reproduction is a genetically programmed event in development that is triggered by an internal signal which shuts down the genes involved in nutrient uptake and activates the conidiation pathway by activating the expression of *brlA* alone. The fungus must somehow sense the external environment and transduce the stimulus (signal) across the cell membrane into the intracellular environment.

7.5 REGULATORY PATHWAY

Having identified the genes which regulate conidiation, the time of their expression could be studied. Total RNA was isolated from cultures and analyzed by Northern blots using *brlA*, *abaA* and *wetA* probes. The experiment detected transcripts of *brlA* at 10 hours, of *abaA* at 15 hours and of *wetA* at 25 hours. The *brlA* mutation blocked the accumulation of all three RNAs. The *abaA* and *wetA* mutations reduced the accumulations of the *brlA* and *abaA* RNAs and blocked accumulation of the *wetA* RNA. The *brlA* and *abaA* mutations affected their own expression and the expression of one another. The *wetA* transcript was absent in *wetA* temperature-sensitive mutants grown at a restrictive temperature, implying that the *wetA* gene is autoregulatory (Mirabito et al., 1989). Subsequently, mutations in six genes were isolated that affect conidiophore development and result in cotton-like colonies with a "fluffy" morphology. These were designated *flbA*, *flbB*, *flbC*, *flbD*, *flbE* and *fluG* and the expression of *brlA* was reduced in these mutants. The non-regulatory developmentally activated genes were divided into early, middle and late depending on the timing of their expression. The three genes *brlA*, *abaA* and *wetA* define a linear dependent pathway (Figure 7.4) in which the activation of *brlA* is sufficient to initiate a cascade of events that involve other genes (Adams, 1995). By examining patterns of RNA accumulation in mutant strains, the developmentally activated genes were divided into four categories (Timberlake and Marshall, 1988; Mirabito et al., 1989). Class A genes are activated by either *brlA* or *abaA* or both, independent of *wetA* (Figure 7.5). *wetA* activates Class B genes, independent of *brlA* and *abaA*. The *brlA*, *abaA* and *wetA* together activate Class C and Class D genes. The accumulation of *wetA* mRNA requires *wetA*$^+$ activity, suggesting that *wetA* is autogenously regulated (Marshall and Timberlake, 1991).

The primary structure of the *brlA* gene was determined and the inferred polypeptide sequence resembled coordination sites that are typical of "zinc finger" DNA binding motifs, indicating that the *brlA* protein is a sequence specific DNA-binding protein—a transcription factor—that activates the expression of a conidiation-specific gene cluster.

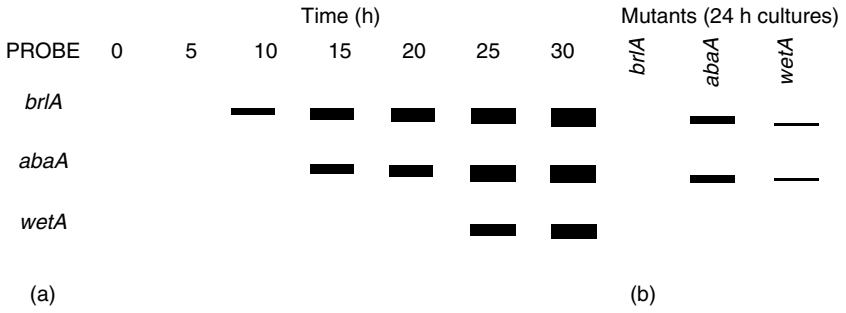

Figure 7.4 Temporal pattern of expression of *brlA*, *abaA* and *wetA* RNAs in *Aspergillus nidulans*. Adapted from Boylan et al. (1987).

Many developmental mutants blocked prior to activation of *brlA* expression were subsequently found. These mutants are described as "fluffy"—they form white, cotton-like aerial hyphae that do not conidiate. These mutants, *fluA*, *fluB*, *fluC*, *fluD*, *fluE* and *fluG*, conidiate when grown on minimal media or when grown in contact with wild colonies, suggesting a signaling molecule is involved in the differentiation of the conidiophore. The signaling molecule can work if the two strains are separated by a dialysis membrane with a 6000 to 8000 dalton pore size, suggesting that it is a low-molecular weight, diffusible compound. The *fluG* (also known as *acoD*) mutant is deficient in the production of a conidiation signal. It is thought that *fluG* participates early, prior to *brlA* in programmed developmental induction pathway (Adams et al., 1998).

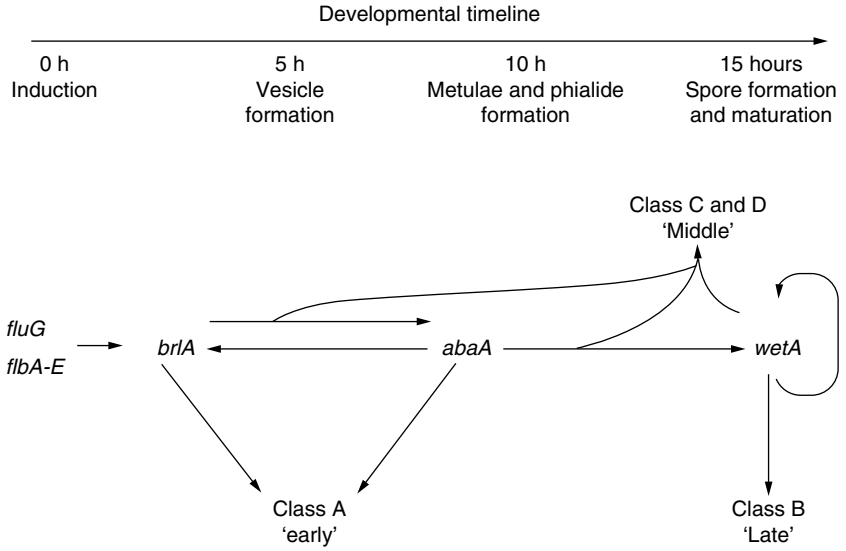

Figure 7.5 Interactions among the regulatory genes during conidiophore development in *Aspergillus nidulans*. (From Adams (1995).)

The work reviewed above shows that the potential of *A. nidulans* is being realized in identifying the key genes that regulate the switch from vegetative growth to the initiation of development that involves hundreds of other genes for the formation of asexual spores.

7.6 SUMMARY

As the mycelium is usually hidden in the substratum, the most conspicuous feature of many fungi is the conidiophore, a device for producing almost countless numbers of mitotically derived spores with the economy of space and time. Characteristically, fungi have airborne spores that serve to disseminate the fungus widely, cause diseases in epiphytotic proportion or cause respiratory diseases as allergens. The conidiophore morphology and modes of development of conidia are an important taxonomic character in the classification of fungi. The synchronized and rapid development of relatively few cell types in *Aspergilllus nidulans*, initiated on schedule and at the correct locations and generating a characteristic form, presents opportunities for the study of eukaryotic development. Since different stimuli are involved in conidiation in different fungi (or in the same fungus), a coordinated signal transduction pathway integrating the perception of stimuli is hypothesized. Genetic evidence from *A. nidulans* shows that asexual reproduction is triggered by environmental factors and conidiophore development occurs in sequence. In response to environmental signals, the mycelium switches to a built-in conidiation pathway involving the transduction of signals to other genes, including *flbB*, *flbC*, *flbD* and *flbE*, that finally results in the activation of the central regulatory gene *brlA*. Three genes, *brlA*, *abaA* and *wetA*, are proposed to define a central regulatory pathway that controls the expression of conidiation-specific genes. These three genes are sequentially expressed and coordinate to direct conidiophore and conidia formation. Among them, *brlA* plays a major role as deduced by the fact that its forced expression is sufficient to direct conidiation. Genes homologous to *brlA*, *abaA* and *wetA* are found in other fungi, indicating that the mechanisms controlling development may be evolutionarily conserved. The complete genome sequence of *A. nidulans* will make possible the determination of the number of genes from the open reading frames. The genes with a role in sporulation can be analyzed using DNA microarrays and can address issues of whether the physical linear order of the genes is related to the time of their expression, the numbers of clusters of co-expressed genes and the effects on gene expression of mutation in a candidate regulatory gene.

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Chapter 8

Ustilago maydis: Mechanisms in Sexual Reproduction

Sexual reproduction in fungi involves the detection of potential mating partners among multiple mating partners present in the environment, coordination of choices by making contact, commitment to a particular mating partner by adhesion, fusion of compatible cells followed by the fusion of gametic nuclei (karyogamy) and the meiotic division of the diploid nucleus to produce haploid progeny in the form of spores. Where the fusing nuclei differ genetically, the haploid progeny have new combinations of genes arising from the assortment of homologous chromosomes and intrachromosomal recombination due to crossing-over. Since the sexually produced spores are often resistant propagules, sexual reproduction could be important for the survival of species in nature where environmental conditions fluctuate.

8.1 HETEROTHALLISM VS. HOMOTHALLISM

The processes leading to fertilization and production of progeny are quite variable in fungi. In the early twentieth century, A.F. Blakeslee found that a large number of morphologically indistinguishable single spore-derived cultures of a species belonging to Mucorales (Zygomycotina) could be sorted into two types, which he called "plus" (+) and "minus" (–) since the strains could not be morphologically distinguished as male or female. If a "plus" colony was grown with another "plus" strain in a petri dish, or if a "minus" strain with another "minus" strain, there was no reaction. However, if the "plus" and the "minus" strains encountered each other, a line of sexually formed spores called *zygospores* formed in the zone of contact. The term *heterothallism* is used to designate when there is the occurrence within a given species of two kinds of individuals, each self-sterile. In this situation, intermycelial reaction is required for sexual fusion, irrespective of the presence or absence of differentiated gametangia (Figure 8.1). From this discovery arose the concept that genes control the mating types. The mating types of strains are designated as *A*/*a* as in *Neurospora crassa* (Chapter 5) or *a*/$\boldsymbol{\alpha}$ in *Saccharomyces cerevisiae* (Chapter 6) to indicate that the differences between the strains are due to two alleles of a single gene.

Conversely, the term *homothallism* is used when a single individual that originates from a single spore forms morphologically similar male and female gametangia and is able to complete sexual cycle, i.e., it is self-fertile and sexually self-sufficient. For example, in the homothallic fungus *Rhizopus sexualis* (Zygomycotina), neighboring hyphal branches of the same individual form gametangia that fuse (Figure 8.2). Here, sexual fusion occurs between

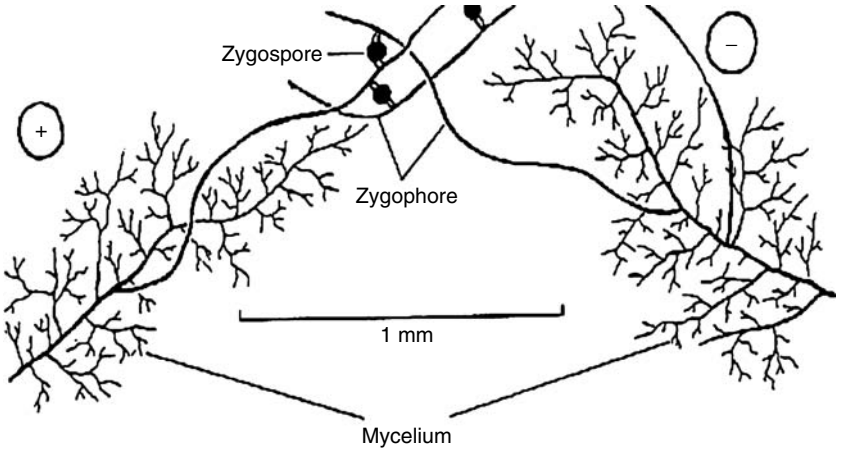

Figure 8.1 Sexual reproduction in heterothallic *Mucor hiemalis* showing mating of morphologically similar cells from two individuals (designated plus and minus) to form zygospore. (From Ingold, C.T. (1961), *The Biology of Fungi*. Chapman and Hall. With permission from Kluwer Academic Publishers.)

cells containing genetically identical (sister) nuclei. Note that although they do not seek a mate, the homothallic fungi still engage in sexual reproduction, retaining karyogamy and meiosis. Since rapid propagation of these fungi can also occur vegetatively, how homothallic fungus benefits from the two processes of karyogamy and meiosis is a puzzling question.

8.2 CELL-CELL RECOGNITION

8.2.1 Mating Types

The fungi grouped in Basidiomycotina are remarkable for having thousands of potential mating partners or mating types. It is of interest to learn how these fungi select a mating partner in the absence of any morphological differentiation. They are remarkable too for having a life cycle in which the nuclei of opposite mating types divide synchronously and remain in close proximity as an $(n + n)$ pair by a special type of hyphal growth called a *clamp connection*, in which a short hyphal branch grows backwards as a hook that fuses with the penultimate cell with the nucleus passing through it such that each binucleate compartment in the hypha acquires nuclei of opposite mating type (see Figure 2.5). The nuclei of opposite mating type in each hyphal compartment remain associated for an extended period of time without fusing—a diploid $(2n)$ phase is postponed in favor of an extended dikaryophase $(n + n)$, the significance of which is a mystery.

Among the fungi in this group used as a model to investigate the mate recognition process are the mushrooms *Coprinus cinereus* and *Schizophyllum commune* and the corn smut fungus, scientifically known as *Ustilago maydis* (Casselton, 2002). Here, we shall highlight the efforts aimed at understanding sexual reproduction in *U. maydis*. This fungus is of interest for other reasons as well: In this fungus, sexual development and pathogenecity are interconnected (Brachmann et al., 2003). Moreover, the fungus exhibits the

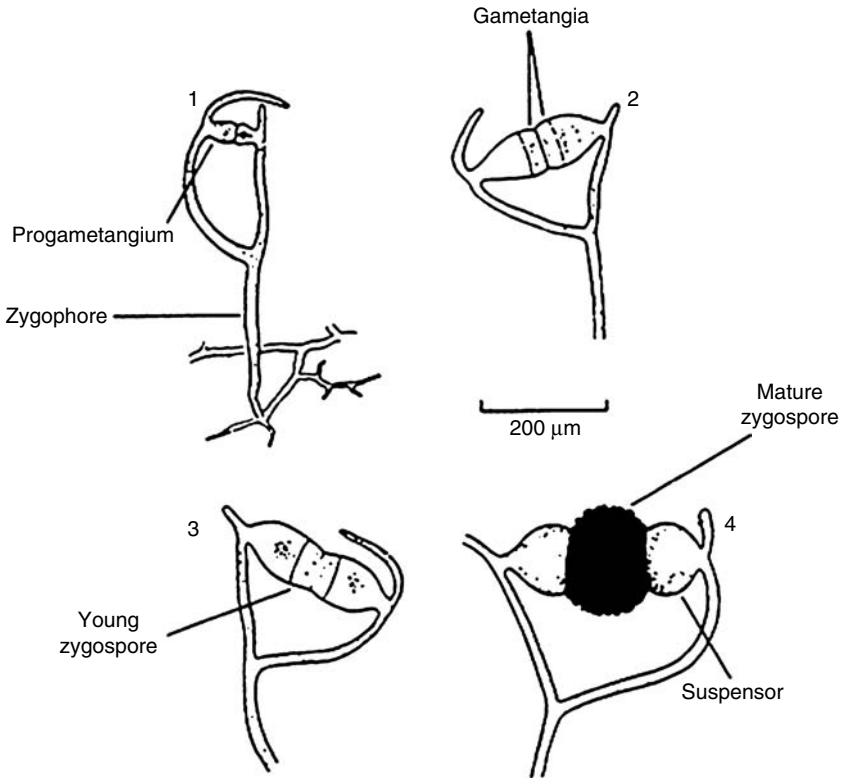

Figure 8.2 Sexual reproduction in homothallic *Rhizopus sexualis*. (1) Mating of a pair of morphologically similar cells or gametangia formed by same mycelium. (2) Delimited gametangia. (3) Fusing gametangia. (4) Mature zygospore. (From Ingold, C.T. and Hudson, H.J. (1993), *The Biology of Fungi*. With permission of Kluwer Academic Publishers.)

phenomenon of dimorphism—it switches from a yeast-like saprophytic form to a filamentous, pathogenic growth. Regine Kahmann's group in Germany is attempting to explain mating and fusion of conjugant cells (sporidia) in biochemical terms and the switch into a pathogenic filamentous mycelium.

U. maydis causes the abnormal growth of kernels or leaves into galls or tumors that are filled with countless numbers of brownish-black spores. These spores are formed by the separation of binucleate hyphal cells and are called *chlamydospores*. The chlamydospores (Figure 8.3) are dormant structures that can survive in crop refuse and in soil for many years. These cells, in which the two haploid nuclei fuse, are also referred to as *teliospores*. The teliospore germinates to form a short tube that becomes divided by septa—a distinguishing structure of fungi belonging to the order Ustilaginales in Phylum Basidiomycotina of the Kingdom Eumycota. This structure is called a *basidium*, in which the diploid nucleus undergoes meiosis and produces four haploid nuclei that are abstricted as basidiospores (also called sporidia). The sporidia can be grown on artificial media where

they multiply by yeast-like budding. The characteristics of the colonies formed from the sporidia of a single basidium differ in color, topography (smooth or rough colonies) and mating type, demonstrating that they are recombinant progeny.

In the first step in sexual reproduction, the cells must distinguish self from non-self and choose a potential mating partner in the environment. The partners must coordinate their choices by making contact, but is the contact based on chance or some physiological mechanism? The sporidia secrete small size polypeptides called *pheromones* that are sensed by sporidia of the opposite mating type in the vicinity, reorient their growth and fuse via conjugation tube. The formation of conjugation tubes using cell culture supernatants is a biological assay for secreted pheromone. An example of visible evidence of cell-cell signaling in a fungus is given in Figure 5.3.

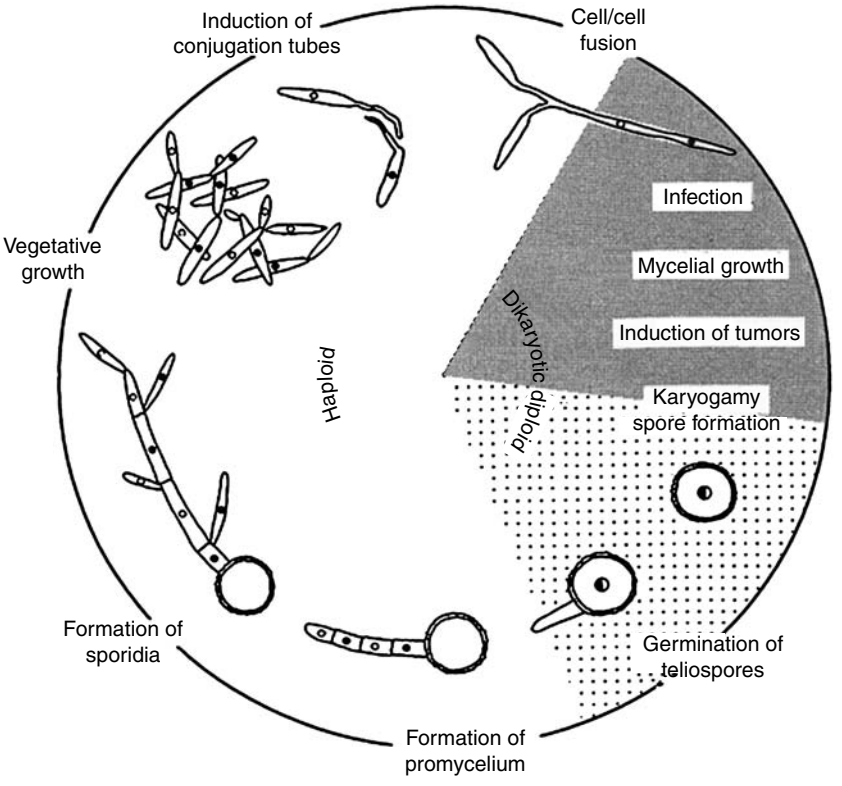

Figure 8.3 Diagram of life cycle of *Ustilago maydis*. The haploid (*n*) sporidia multiply to form secondary sporidia by budding. Sporidia of compatible mating type fuse to form a dikaryotic mycelium ($n + n$) that infects a corn plant, the kernels of which are filled with powdery mass of spores (chlamydospores). Nuclear fusion occurs in chlamydospores (teliospores) that are 7–10 μm in diameter. These germinate to form a tube-like basidium in which the diploid ($2n$) nucleus divides by meiosis. The four haploid nuclei are abstricted in four cells (sporidia) which can grow saprophytically on refuse and multiply by budding. (From *The Mycota* I, Mating-type genes in heterobasidiomycetes, Kämper, K., Bölker, M. and Kahmann, R., p. 326, Fig. 2, 1994, © Springer-Verlag Berlin Heidelberg. With permission.)

A simple test of mating compatibility is to co-spot sporidia on a nutrient agar medium containing charcoal where the fused sporidia show filamentous growth (the "fuz" reaction). However, the sustained growth of the hypha can occur only as a parasite in the corn plant (*Zea mays*). Only the haploid cells (sporidia) that differ at two mating type loci can mate, resulting in hypha (dikaryon) in which the two nuclei divide several times but maintain their nuclear identity. Fertilization is postponed in favor of dikaryosis. This mycelium is a special type of heterokaryon in which each cellular compartment contains two nuclei of opposite mating type in close proximity without fusion. The dikaryotic hypha recognizes and enters into the corn plant, which responds to infection by forming galls (tumors) filled with chlamydospores (teliospores).

8.3 EXTRACELLULAR RECOGNITION

8.3.1 The *a* Locus

The fusion of sporidia is controlled by two unlinked mating type loci, designated as *a* and *b*. Compatible mates have different alleles of genes at both loci. The *a* mating type locus has two "alleles," *a1* and *a2*, that are structurally quite dissimilar even though they are at the same position in the chromosome. This unusual situation of dissimilar mating type genes being present at the same locus in the chromosome is distinguished from the term "allele" by the term *idiomorph*. The idiomorphs are responsible for extracellular cell-cell recognition and the fusion of sporidia (Figure 8.4).

To understand the mating process at molecular level, Regine Kahmann's group cloned and determined the DNA sequence of *a* and *b* mating type loci and performed functional assays based on transformation.

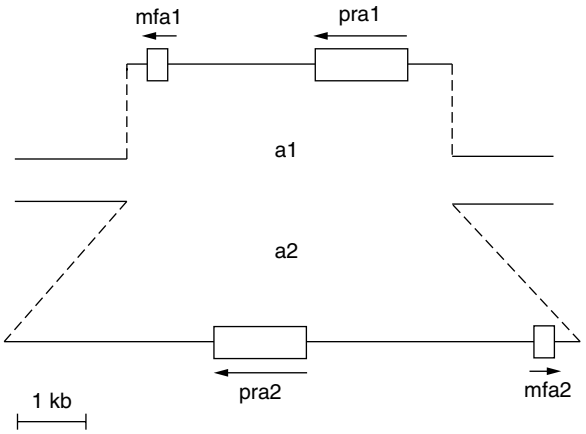

Figure 8.4 Schematic representation of the *a* idiomorphs. The *a* alleles have little sequence homology but occupy the same chromosomal location. *mfa*, pheromone precursor gene; *pra*, receptor gene. Arrows indicate the direction of transcription. (From *The Mycota* I, Mating-type genes in heterobasidiomycetes, Kämper, K., Bölker, M. and Kahmann, R., p. 326, Fig. 2, 1994, © Springer-Verlag, Berlin and Heidelberg. Reprinted with permission.)

8.3.2 Pheromone and Receptor

The cloning strategy of the *a* locus is based on the observation that when both *a1* and *a2* alleles are introduced by transformation in the same cell, *U. maydis* behaves as a "double mater," i.e., it can mate with strains of either *a* mating type (Figures 8.5 and 8.6). The

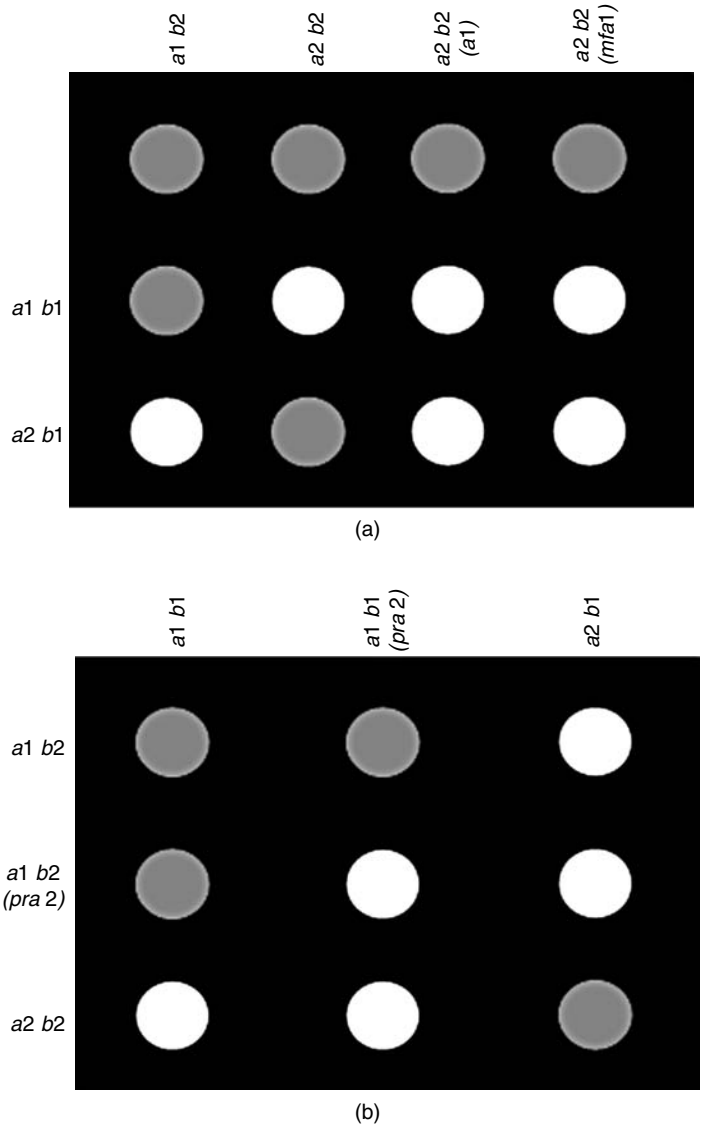

Figure 8.5 Diagram of test for mating reaction in *Ustilago maydis* on charcoal-containing medium. Cells were cospotted as indicated on the top and on the left. Genes introduced by transformation are in parenthesis. (a) Double mater phenotype of *a2b2* transformed with *a1*. (b) *a1b1* and *a1b2* were transformed with receptor gene *pra2*. Compatible cells produce white-filament (fuz^+ phenotype). Based on Bölker, M., Urban, M. and Kahmann, R. (1992). *Cell* 68: 442–450, © 1992. With permission from Elsevier.

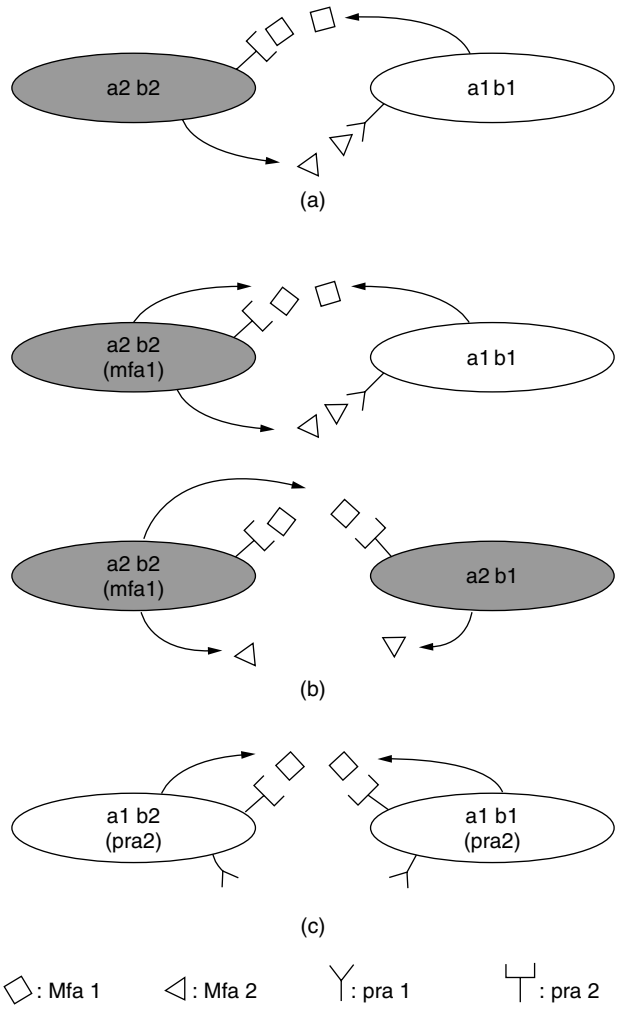

Figure 8.6 Schematic representation of interactions between pheromones and receptor coded by the *a* locus of *Ustilago maydis*. (a) Mating interaction between compatible cells differing at both *a* and *b* loci. (b) Double mater phenotype of *a2* strain transformed with the pheromone gene from the opposite *a* mating type. Mating occurs between haploid strains of both *a* mating types. (c) Strains of the same *a1* mating types can fuse if both are transformed with the receptor gene (*pra2*) from the opposite mating type. *mfa* and *pra* introduced by transformation are denoted in parentheses. Reprinted from *Cell* 68, Bölker, M., Urban, M. and Kahmann, R. (1992), pp. 442–450, © 1992. With permission from Elsevier.

nucleotide sequence of a loci revealed that they encode small size polypeptides with a carboxy-terminus sequence of Cys-A-A-X, where *A* is an aliphatic amino acid and *X* any amino acid with a farnesyl group attached to cysteine—a motif that is characteristic of pheromones (Figure 8.7). Moreover, the nucleotide sequencing data disclosed two genes called *pra1* and *pra2* that encode proteins having seven hydrophobic, potential membrane-spanning

mfa1 MLSIFAQTTQTSASEPQQSPTAPQGRDNGSPIGYSSCVVA
mfa2 MLSIFETVAAAAPVTAETQQASNNENRGOPGYYCLIA

Figure 8.7 *Ustilago maydis*. Polypeptide sequences encoded by *mfa1* and *mfa2*. Amino acids are shown by one-letter symbols. The Cys-A-A-X motif at the C-terminal end is underlined. After Bölker et al. (1992).

regions, a characteristic feature of the receptors found in the membrane. From this it is inferred that *a* locus encodes a pheromone polypeptide and a receptor; it is the primary determinant of cell-cell recognition. Mating of *a1* and *a2* compatible cells occurs if the *mfa1* receptor recognizes the *a2* pheromone and the *mfa2* receptor recognizes the *a1* pheromone. Haploid strains of the same *a1* mating type fuse if both are transformed with the receptor gene (*pra2*), provided they contained the different *b* allele.

8.4 INTRACELLULAR RECOGNITION

8.4.1 The *b* Locus

8.4.1.1 Regulatory Function of *b* Polypeptides

After recognition of mating partners through a system based on pheromones, further sexual development occurs through a multiallelic *b* locus which determines whether a filamentous, pathogenic dikaryon is formed or not. For fusion of sporidia to occur, the nuclei must have two different *b* alleles (Table 8.1). Thirty-three different *b* alleles (*bE* 1 to 33 and *bW* 1 to 33) have been identified. This means that a system of recognition of nuclei exists by which any combination of $33^2 - 33$ or 1056 different *b* alleles are active and 33 combinations of the same allele are inactive (Kämper et al., 1995). The clue to this system emerged from the cloning of the *b* locus. The cloning strategy for the *b* locus is based on the observation that the diploid strain *a1a2 b1b2* strain develops fuzziness (limited filamentous growth) on artificial media containing charcoal. The introduction by transformation of the *b* allele in a diploid cell that is heterozygous for *a* but homozygous for *b* gives the "fuz," indicative of a mating reaction. The fuz will induce tumors if introduced in a corn plant; therefore, the fuz reaction is a valid test of successful mating. The fuz reaction allows the cloning of the *b* locus and determination of its nucleotide sequence.

Table 8.1 Mating Reaction of Sporidia of *Ustilago maydis*

Genotype	*a1b1*	*a1b2*	*a1b3*	*a2b1*	*a2b2*	*a2b3*
a1b1	–	–	–	–	+	+
a1b2	–	–	–	+	–	+
a1b3	–	–	–	+	+	–
a2b1	–	–	–	–	–	–
a2b2	+	–	+	–	–	–
a2b3	+	+	–	–	–	–

Note: + = Successful mating reaction results in formation of a dikaryotic mycelium (fuz reaction). The *a* locus is biallelic and the *b* locus is multiallelic; however, only three are shown.

The *b* locus contains a pair of divergently transcribed genes, *bE* (east) and *bW* (west), separated by a spacer region (Kämper et al., 1995). From the DNA sequence information, *b* polypeptides were predicted to have a constant and a variable domain and a homeodomain motif. Homeodomain proteins, first identified as products of *Drosophila* genes with an important role in embryonic development, bind to DNA and function as transcription factors regulating development. A yeast two-hybrid system (Chapter 6) was used to demonstrate that *bE* and *bW* proteins can associate into a dimer but only if they are derived from different alleles. It is postulated that only the pair-wise combinations of specific *bE* and *bW* that originate from different alleles of the locus interact through complementary hydrophobic sequences to form heterodimers (Kämper et al., 1995; Kahmann and Bölker, 1996). There is no interaction between proteins if they are encoded by the same mating type—self-nonself recognition is at the level of protein dimerization (Figure 8.8). Thus, no single strain is self-fertile. It is expected that we shall learn to what regulatory DNA sequences the combination of polypeptide encoded by one *bE* and one *bW* gene bind, what these transcripts code for

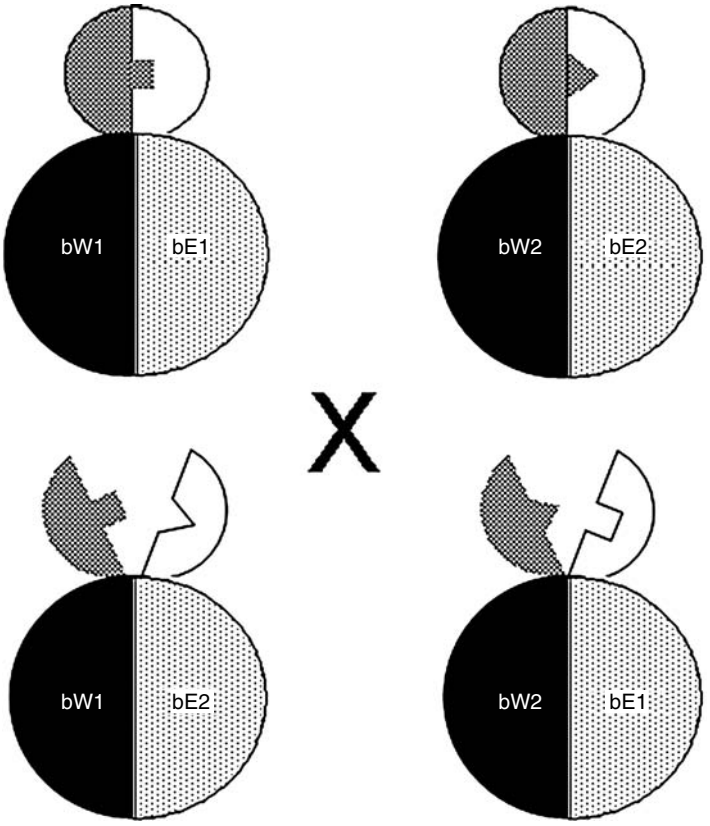

Figure 8.8 *Ustilago maydis*. A schematic model proposed for allele-specific dimerization. The interaction of *bE* and *bW* polypeptides from the same *b* allele produces inactive heterodimers because the DNA-binding domains are buried. Upon mating, two additional new types of active heterodimers are formed in which DNA-binding motifs are exposed. (From Gillissen et al. (1992). With permission of the Cell Press.)

and how the cascades of signal transduction lead to pathogenic growth in the corn plant leading to the fusion of the nuclei in the dikaryon and the completion of the life cycle.

8.5 OVERVIEW

Morphological specialization (production of differentiated gametangia) has little relevance to mating in fungi based on the haploid phase. Indeed, specialized cells for mating are found only in filamentous Ascomycotina and even here a single individual produces both male and female structures. Nonetheless, most species have genetic barriers to self-fertilization and only individuals with different mating types can engage in sexual reproduction. Ascomycotina have just two mating types but Basidiomycotina may have several hundred mating types. The mating type loci encode proteins and different mating types bring compatible versions together. Only proteins from different mates that interact with one another can activate sexual development and promote outcrossing. Sexual reproduction guarantees genetic variability in a species. The genes that determine fungal mating type are located at one or more complex loci.

U. maydis exemplifies several features of sexual development also found in the familiar mushroom fungi. In the absence of morphological differentiation of gametangia, recognition of conjugants is based on pheromone-based recognition. There are hundreds of different genetically determined mating types (individuals). The *a* locus has two known mating types with each allele containing two genes, one for a pheromone polypeptide and one for a pheromone receptor. The necessary condition for a successful mating is that two conjugant partners must be complimentary. Self-nonself recognition is at the level of protein dimerization. The significance of fertilization (diploidy) being postponed in favor of an extended dikaryotic phase is elusive. Despite the lack of morphological differentiation in fusing partners, fertilization and meiosis are still involved. Sexual fusion reprograms development—the mating of two compatible haploid sporidia switches to a filamentous form that is pathogenic. Elucidating the cascades of the signal transduction pathway into distinct cellular responses, such as the decision to grow as yeast, engage in mating, or colonize a maize plant, is the focus of current work.

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Part IV

Gene Manipulation in Fungi

Chapter 9

Transformation of Fungi and Discovery of Gene-Silencing Phenomena

> As is the way of scientific progress, once one issue is resolved, other, more intriguing, unexpected problems are revealed.
>
> *John H. Burnett*

In 1928, F. Griffiths discovered an ingredient in heat-killed cells of the pathogenic bacterium *Streptococcus pneumoniae* that could transform a live, non-pathogenic mutant strain of the bacterium into a pathogenic strain. In 1944, O.T. Avery and his colleagues identified the transforming principle in the cell-free extracts as DNA. The introduction of a piece of a homologous or foreign DNA molecule into an organism such that it is stably integrated into the host genome and stably alters its character is one of the most powerful tools in biology. It allows genes to be cloned through their ability to complement mutant phenotypes and to study their functions by selective knock out, the replacement of an endogenous gene with an engineered derivative.

From both basic reasons and practical benefits, it is of interest to determine the effects of introducing and integrating extra copies of a gene into the genome of a cell. The genes controlling pigmentation in *Neurospora* (Figure 9.1) or flower color in plants are especially useful as visual reporter systems to study the expression of transgenes, e.g., will the color be intensified? The unexpected finding, however, was that often the color is suppressed or extinguished. Such observations in fungi and plants led to the recognition of the phenomena of gene-silencing. This is considered to be a defense mechanism that evolved early for the preservation of the integrity of the genome from the onslaughts of viruses and transposons.

Because fungi reproduce rapidly and do so both sexually and asexually, they are particularly attractive for the study of gene-silencing phenomena in the vegetative and sexual phases. Additionally, the ability to fuse their vegetative cells allows the study of the dominance or recessiveness of the transgene in heterokaryons (Section 9.4.4). These experimental possibilities brought fungi to the forefront of research in this area.

9.1 TRANSFORMATION PROCEDURE

Methods were developed for transferring DNA into fungal cells and to select the transformed cells that have taken up this DNA. In the prototype experiment done in Edward Tatum's laboratory, wild type DNA was introduced into an inositol-requiring mutant

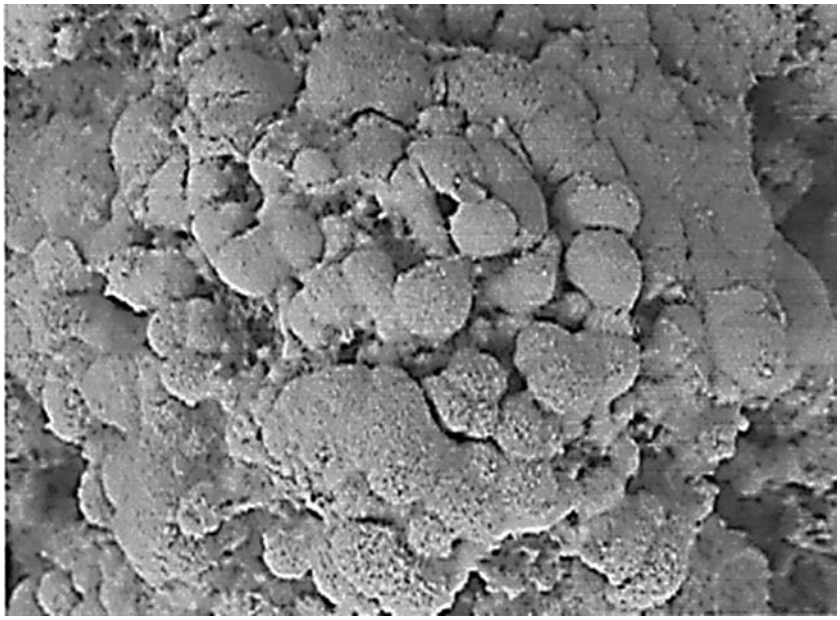

Figure 9.1 *Neurospora crassa* growing on a sugarcane factory-waste dump. The genes encoding pink-orange color have been used as a visual reporter system in gene-silencing experiments. (Photo: courtesy of P. Maruthi Mohan.) (See color insert following page 140.)

(auxotroph) of *Neurospora crassa*. The use of an auxotrophic mutant as the recipient allowed the rare, inositol-independent cells (prototroph) to be directly selected on minimal media lacking the nutritional supplement. However, the prototrophic selection method has the disadvantage that the nutritional mutation has to be introduced into the recipient (host) strain by a prior sexual cross. This time-consuming difficulty is overcome by the use of a dominant selectable marker (Hynes, 1996), for example, the mutant β-tubulin gene (*Bml*^{R}), which confers resistance to the fungicide benomyl, or the hygromycin resistance gene (*hph*) which allows transformants to be selected even in the wild-type background. The DNA-treated conidia are plated on media that contains benomyl or hygromycin on which the untransformed cells cannot grow, allowing the transformed colonies to be selected.

The entry of DNA into cells is facilitated by removal of the cell wall. The cell wall can be removed by treating cells with a commercial mixture of β, 1-3 glucanase and chitinase enzymes (Novozyme) obtained from a soil fungus, *Trichoderma viride* (see Davis, 2000). The protoplasts (spheroplasts), freed from the constraint of the rigid cell wall, become round and are prone to bursting in a hypertonic environment and, therefore, need to be osmotically stabilized to regenerate a mycelial culture. Because the commercial preparations of the lytic enzymes vary from batch to batch, the protoplasting method yields variable results; it was therefore replaced by the electroporation or the particle bombardment methods for introducing DNA. In the now commonly used electroporation method, conidia are placed in a solution of DNA and subjected to strong electric pulses to facilitate the entry of DNA into the cell, presumably by transiently opening holes in the membrane.

In practice, the DNA to be used for transformation is cut into fragments by DNA-cutting enzymes called *restriction enzymes*. A fragment is joined into a small circular DNA

molecule called a plasmid (vector) containing a point (restriction site) for joining the foreign DNA, one of which will have the gene of interest. The vector DNA selected is one that contains a selectable marker and regulatory sequences necessary for the expression of the transgene. The ligated, recircularized DNA fragment containing the gene of interest is introduced into the fungal cell (generally conidia) by transformation. Typically, 1 to 20 μg of DNA per 10^7 cells in 50 μl of a pH 7.5 buffer containing 1M sorbitol or polyethylene glycol 4000 and 50 mM of $CaCl_2$ are incubated before plating the conidia on selective agar media to select the rare, specific colonies of transformed cells. The transformation frequency is about 1 to 20 per microgram of DNA. The specific fragment of DNA from a single chosen transformed colony can be reisolated (cloned) by taking advantage of the fact that it is now tagged with DNA sequences of the transformation vector.

9.2 HOMOLOGOUS VS. ECTOPIC INTEGRATION OF TRANSGENE

In contrast to yeast, where the transforming DNA molecule commonly integrates at the related sequence by cross over (homologous recombination), integration in the filamentous fungi occurs commonly at unrelated sequences (heterologous or ectopic recombination). As an example, Figure 9.2 shows the partial restriction map of a circular plasmid carrying

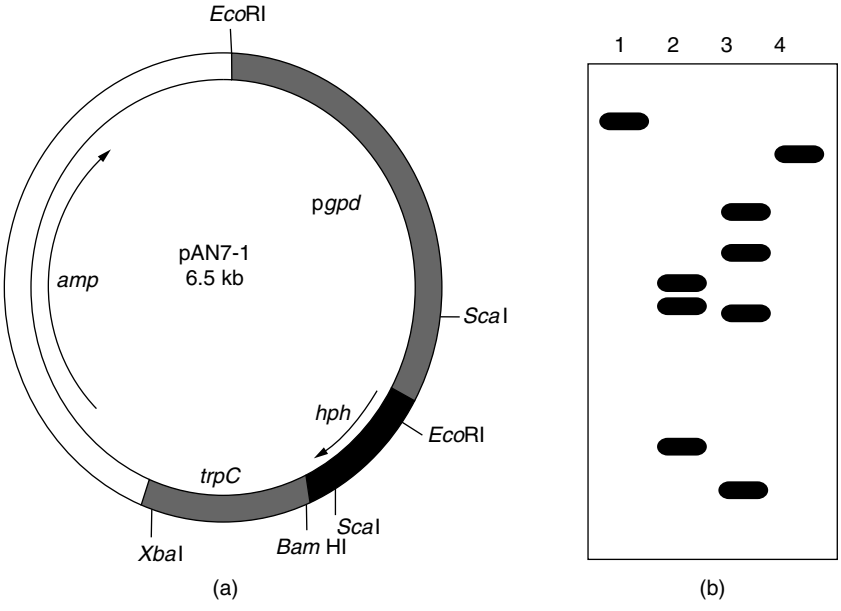

Figure 9.2 (a) Map of plasmid used to obtain Hyg^R transformants of *Ascobolus immersus*. Black thick lines correspond to *hph* genes, dotted lines are regions containing regulatory elements *gpd* promoter and *trpC* terminator from *Aspergillus nidulans* and restriction sites for *XbaI* (Xb), *BamH1* (B), *EcoR1* (E) and *Sca1* (Sc), ampicilllin resistance (amp). (b) Southern hybridization analysis of transformants using a *hph* probe corresponding to a Sca1-Sca1 fragment. Transformants (lane 1 and 4) have integrated one copy of the transgene. Transformants (lane 2) and (lane 3) have integrated three and four copy of the transgene, respectively. Based on Rhounim et al. (1994).

the selectable marker gene, hygromycin phosphotransferase gene (*hph*) from *Escherichia coli* and the control regions (promoter and terminator). This vector was used to transform *Ascobolus immersus* (Rhounim et al., 1994). On average, the transformation frequency is 25 per μg of plasmid DNA. The recipient may be transformed by one or more ectopic integrations of the plasmid DNA at random sites in the genome (ectopic integration). The mode of integration is determined by Southern blot analysis of transformants (Figure 9.2b). Transformants, which have integrated only one copy of the transgene, will exhibit only one *XbaI* band (lane 1 and lane 4). Multiple integrations are seen in transformants in lanes 2 and 3, which have three and four integrations, respectively. Note that the *XbaI* site is located in the 3 flanking sequence of the *hph* gene. The number of *XbaI* fragments will correspond to the number of transgenic copies if the multiple integrations are dispersed in the genome. This will also be seen if the multiple copies integrate in tandem at a single site.

Transformation is now routinely done in several fungi (Fincham, 1989; Hynes, 1996). Chapter 8 illustrated the use of transformation in understanding the mating process in the pathogenic fungus *Ustilago maydis*; here, the bias is heavily toward *Neurospora crassa*.

9.3 PURIFICATION OF TRANSFORMANT

Purification of the transformed nucleus is facilitated in those fungi that form uninucleate conidia—for example, *Aspergillus* (Chapter 7)—or that can be manipulated to selectively produce uninucleate cells, as with *N. crassa* (Maheshwari, 2000). In *N. crassa*, macroconidia are easily obtained and are therefore routinely used for transformation. However, as these cells are usually multinucleate, the transforming DNA integrates at random locations in the genome and in different numbers of copies in different nuclei. Since not all nuclei in the same cell are transformed similarly (Pandit and Russo, 1992; Groteleuschen and Metzenberg, 1995), the transformants are commonly heterokaryotic. Thus, a single transformed nuclear type needs to be purified. During macroconidia formation, nuclei from the mycelium enter into macroconidia in varying numbers, generally between one and four. The primary transformants are heterokaryotic, which requires the purification of a transformant having a single nuclear type. This is done by the rather time-consuming method of repeatedly plating a dilute suspension of macroconidia in series and picking up single colonies showing the transformed phenotype. Alternatively, a genetic method can be used to purify a transformed nucleus from a heterokaryotic transformant by crossing it to an untransformed strain and selecting the transgene-bearing segregants among the meiotic progeny. However, purification of the transformed nucleus by the crossing method has generally been unsuccessful because very often the transgene is not transmitted to the progeny.

9.4 GENE-SILENCING PHENOMENA

9.4.1 Silencing by Mutation (RIP)

Selker et al. (1987) investigated the underlying reason for the non-inheritance of transgenes in *Neurospora*. Specifically, they determined the fate of transforming DNA carrying single and duplicated DNA sequences and sequences that are normally unmethylated or methylated (Figure 9.3). A vector was constructed that had a single copy of the am^+ gene and

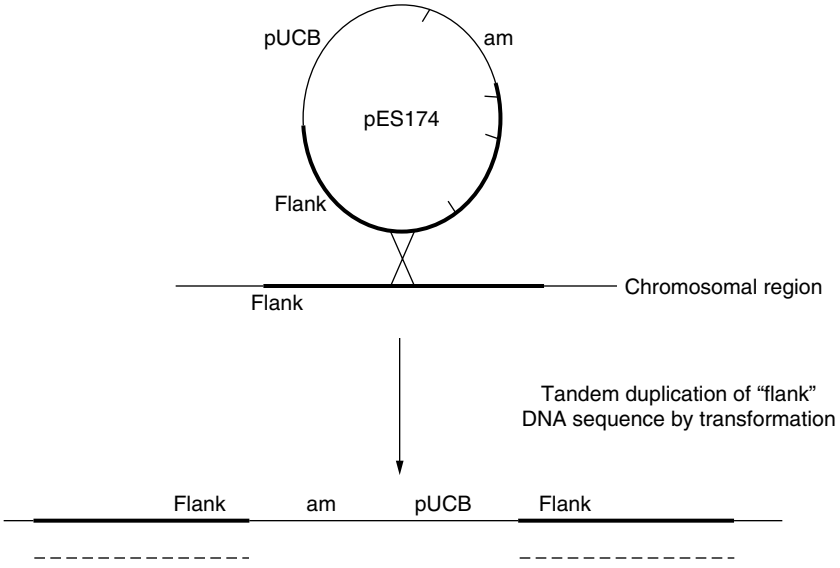

Figure 9.3 Selker et al.'s (1987) method of generating linked duplication of DNA sequence in *N. crassa* by homologous recombination of a plasmid. The only homologous sequence between pES174 and host was a 6 kb "flank" region (indicated by heavy line). The fate of this duplicated sequence between fertilization and nuclear fusion was studied.

a duplicated am^+ flank region. This plasmid vector containing the wild type (am^+) was used to transform an *am* (glutamate dehydrogenase) deletion mutant of *Neurospora crassa* to study the fate of a single copy of the am^+ gene and a duplicated am^+ flank region. The transformant was crossed to a strain lacking the *am* gene and the ascospore progeny were analyzed by Southern hybridization using the *am* gene probe to determine if they had inherited the transgene. The progeny showed a 10 kb band (Figure 9.4) that was not present in the primary transformant. This novel band could result only if the *BamHI* restriction sites *b*, *c* and *d* in the duplicated region had become modified. (*BamHI* is a restriction enzyme obtained from *Bacillus amyloliquifaciens*; it differs, for example, from *EcoRI*, a restriction enzyme from *Escherichia coli*.) By using a pair of restriction enzymes that distinguish methylated and unmethylated DNA (isoschizomers), the cut DNA upon electrophoresis shows a new band due to a change in the restriction sites by the modification of the cytosine residues in the DNA sequence by methylation of only the duplicated *am* flank region. The process that altered the extra (foreign) DNA sequences by mutations and epigenetic modification of the cytosine residues in DNA by methylation was named RIP, an acronym for *repeat-induced point* mutations. By dissecting out and analyzing ascospores meiotic tetrads (8-spored progeny asci), the timing of RIP could be determined by analyzing the meiotic tetrad. It was inferred that RIP detects and mutates duplicated sequences during pairing of homologous chromosomes prior to karyogamy in the ascus initial. RIP depends upon the capacity of premeiotic cells to recognize the presence of duplicated sequences in an otherwise haploid genome (Figure 9.5). The only copies of

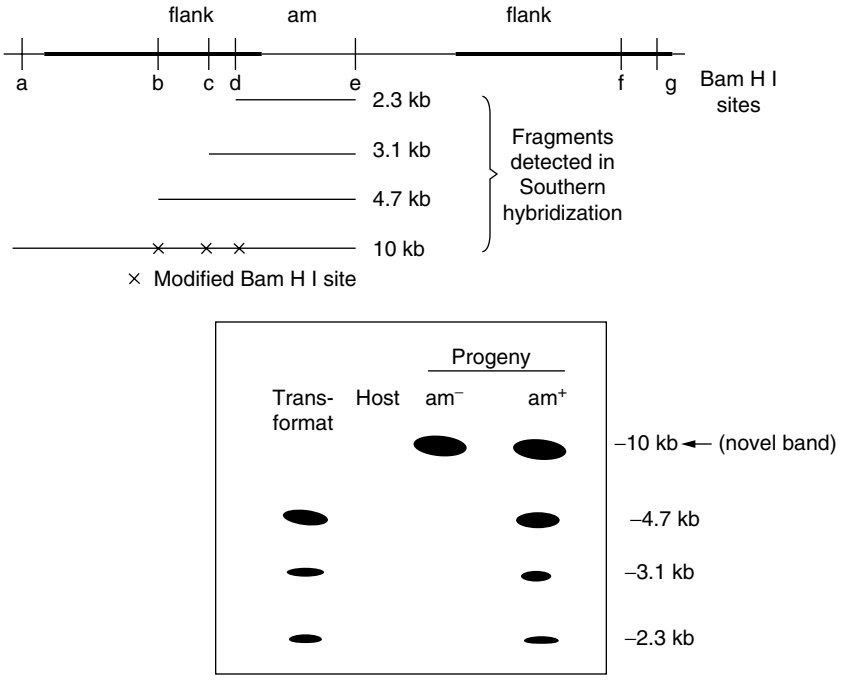

Figure 9.4 Discovery of RIP phenomenon. Detection and modification of transforming sequences in the *am*$^+$ progeny from the cross of transformant *X* host DNA was digested with *BamHI*, fractionated and probed with the *am* region. The appearance of a novel 10 kb band in the *am*$^-$ progeny indicated modification or deletion of *BamHI* sites *b*, *c* and *d* in the duplicated regions of the progeny. The *am*$^+$ progeny retained all four regions of the plasmid. Based on Selker et al. (1987).

genes immune to RIP are the tandemly repeat units of rDNA, presumably protected because of their localization in nucleolus.

9.4.2 Meiotic Silencing by Unpaired DNA (MSUD)

In *N. crassa*, mutation in the gene *Asm-1* (*ascus maturation*) affects protoperithecial formation. When a deletion mutant, designated *Asm-1*, was crossed to wild *asm-1*$^+$, the ascospores formed were all unmelanized and inviable (Aramayo and Metzenberg, 1996; Shiu et al., 2001). The defect was corrected by transformation of *Asm-1* by a cloned *Asm-1*$^+$ gene. Interestingly, the cross of two deletion mutants yielded black ascospores. Analysis of several crosses (Figure 9.6) involving the deleted *asm-1*$^+$ gene and copies of normal genes in normal or ectopic locations led to the conclusion that the presence of an unpaired copy of *asm-1* silenced the expression of all copies of *Asm-1*, whether paired or unpaired. This gene silencing phenomenon was called *meiotic silencing of unpaired DNA* (MSUD). Thus, whereas RIP scans the genome for duplicated sequences before karyogamy, MSUD operates after karyogamy, recognizing any unpaired DNA sequences and silencing them. This gene silencing mechanism might be important in holding down the genetic load due to transposable elements that move during meiosis.

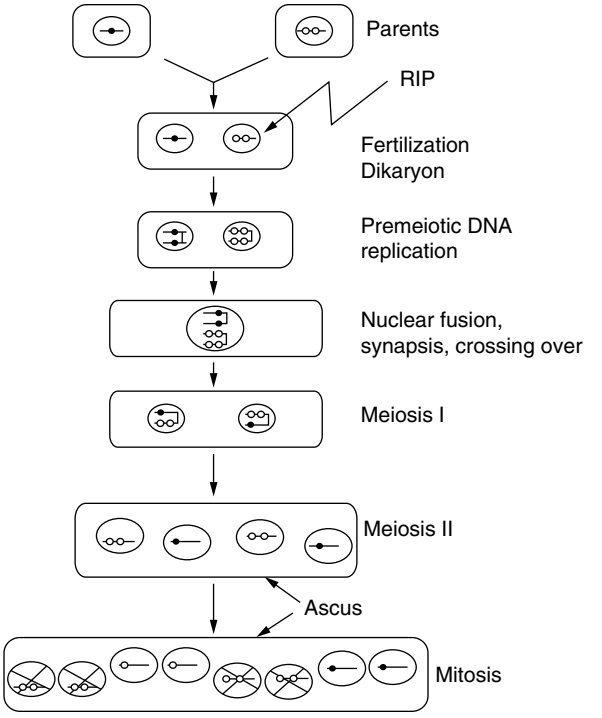

Figure 9.5 A diagram showing timing and consequence of RIP. Based on Selker et al. (1987).

9.4.3 Silencing by DNA Methylation (MIP)

We consider two examples of reversible inactivation of transgenes by cytosine methylation. In *Ascobolus immersus*, the transformation of a methionine auxotroph (*met-2*$^-$) by a plasmid carrying *met-2*$^+$ allele resulted in its integration (Goyon and Faugeron, 1989). When the transformant carrying an extra copy of a gene was crossed to a wild type, both the normal and the ectopic copies were inactivated. Since both copies of each allele in the meiotic tetrad were inactivated, the inactivation must occur before the premeiotic chromosome division. The frequency of inactivation of the transforming DNA was doubled if the *met-2*$^-$ genes are repeated in tandem than if the duplicated genes are at the ectopic site. No point mutations are associated with methylation. Gene inactivation is spontaneously reversible; where the progeny have a *met-2*$^-$ phenotype (Faugeron et al., 1989), the reversion rate is increased by growing the fungus in the presence of 5-azacytidine, an analog of cytidine that prevents cytosine methylation, suggesting that methylation plays a major role in this inactivation and accompanying gene silencing. This phenomenon is called MIP for *methylation induced premeiotically.*

In another study, *N. crassa* was transformed with the hygromycin phosphotransferase (*hph*) gene (Pandit and Russo, 1992). When the primary (heterokaryotic) transformant having more than one copy of the transgene was grown in the absence of hygromycin, the expression of the hygromycin (*hph*) transgene was silenced, as determined by the very low percentage of colonies formed from conidial plating on hygromycin supplemented

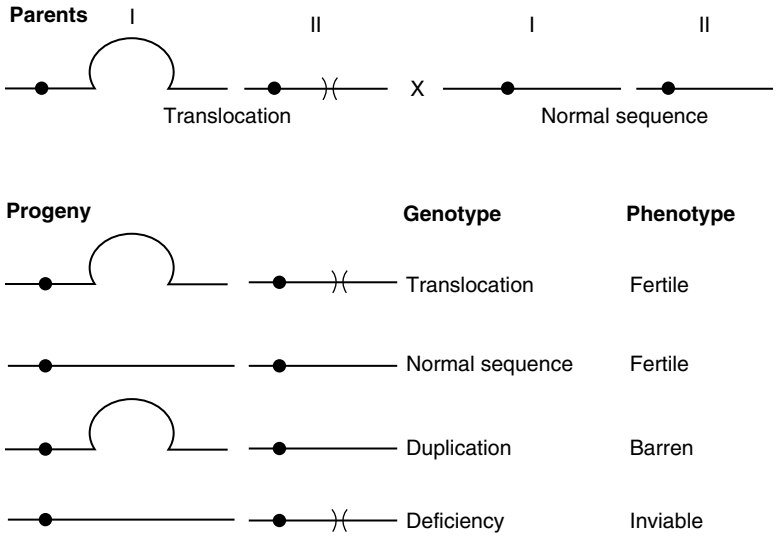

Figure 9.6 Diagram of *Neurospora crosses* that led to discovery of MSUD. Only two linkage groups are shown. The translocation is indicated by inverse parentheses that is indicated by looping out. Crosses between normal sequence strains and translocation strains yield three kinds of progeny: normal sequence (like one parent), translocation sequence (like the other parent) and a novel class that is duplicated for the translocated segment. The duplication progeny arise when one component of the translocation segregates with a normal sequence chromosome; deletion progeny resulting from segregation of the complimentary component are inviable. Duplication strains produce perithecia that lack ascospores (barren phenotype). (From Kasbekar, D.P (2002). *J. Biosci*, pp. 633–635.)

(+Hyg) media. Silencing is reversed if the transformant is grown in the presence of hygromycin (Figure 9.7) or 5-azacytidine, an inhibitor of methylation. This observation suggests that methylation plays a role in the reversible silencing of the *hph* gene.

9.4.4 Quelling

The characteristic orange color of *N. crassa* conidia is due to carotenoid pigments produced from mevalonic acid by a series of reactions that involve dehydrogenation, cyclization and cis-trans isomerization (Figure 9.8). Three mutations are known which block different steps in carotenoid biosynthesis. Any of these three mutations block the production of pigment, resulting in an albino (white) phenotype. The three genes were named *al-1*, *al-2* and *al-3* in the order of their discovery and all three genes have been cloned and sequenced.

In an attempt to overexpress carotenoid, Romano and Macino (1992) transformed a wild, orange colored strain with the wild *al-1*$^+$ gene. Unexpectedly, the color in transformants was extinguished or suppressed—the transformants were white or pale yellow. Similarly, the duplication of chalcone synthase gene (*chs*) involved in anthocyanin in flower petals results in the loss of flower color in plants where this phenomenon is known variously as co-suppression, repeat-induced gene suppression or homology-dependent

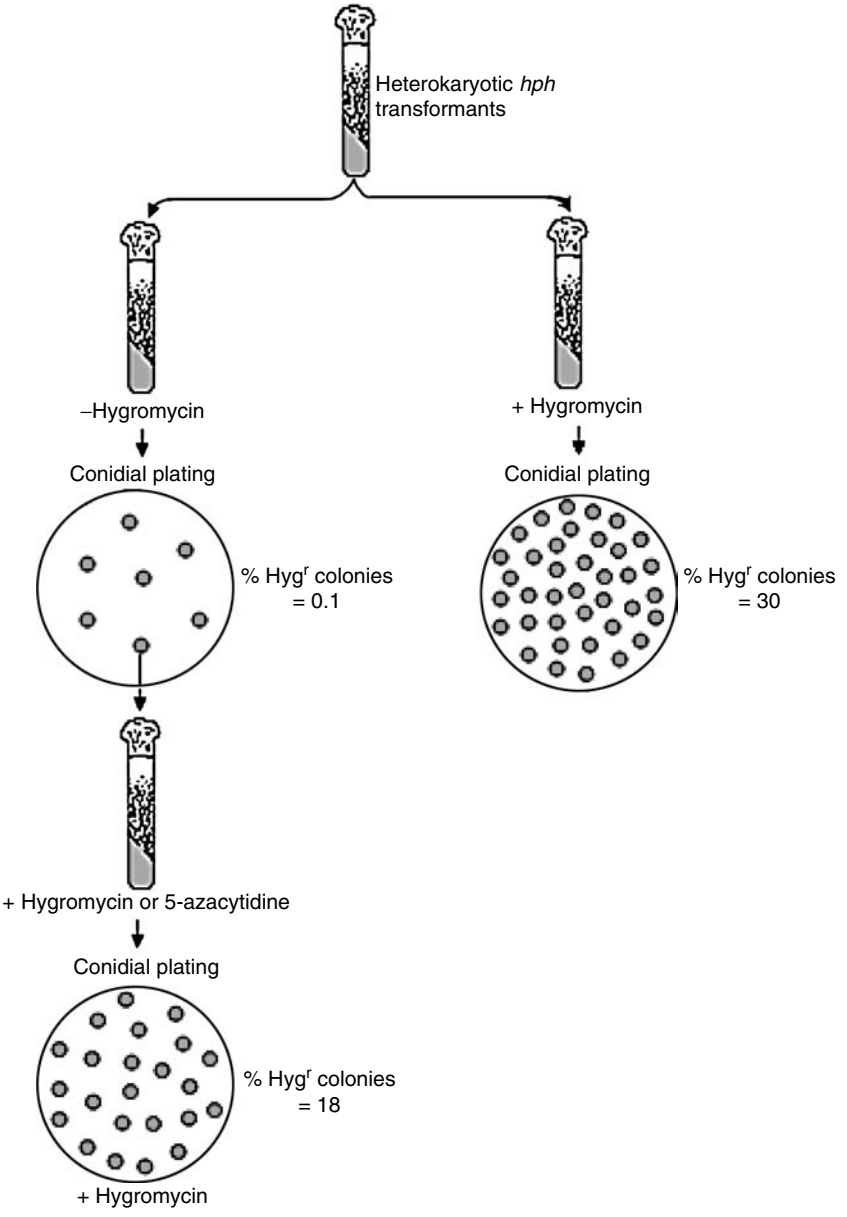

Figure 9.7 Diagram of silencing of *hph* transgene in *Neurospora crassa*. Based on Pandit and Russo (1992).

gene silencing. These terms all mean the same thing, i.e., the mutual inactivation of gene expression by homologous sequences. The phenomenon has received more attention and publicity in plants because the expression of transgenes is necessary for crop improvement. However, *Neurospora* has provided the best clues because of the opportunity of investigating the involvement of a trans-acting diffusible molecule using heterokaryons. The

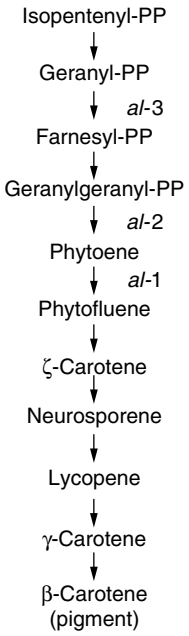

Figure 9.8 Carotenoid biosynthesis pathways showing the steps blocked in albino mutants.

albino genes of *Neurospora* provide a visual reporter system for identifying the silenced strains that have an albino phenotype (Romano and Macino, 1992).

The phenomenon of silencing of the endogenous (resident) and the transgene copies of genes in the vegetative phase was termed *quelling*. Transformation of the wild *Neurospora* strain with different portions of the *al-1* gene shows that a minimum of about 130 bp of coding region can induce quelling. As this size would be insufficient to code for a functional protein, the requirement of transgene protein for quelling was ruled out. If the transforming DNA was *al-3*, Northern blot analysis shows that *al-3* mRNA is absent, not *al-1* or *al-2* mRNA. The transcriptional silencing of homologous endogenous genes is the hallmark of gene-silencing events in fungi or plants. But whereas in plants almost all cases of gene silencing are associated with the repression of transcription due to the methylation of cytosine residues in the resident as well as the transgenes, quelling in *Neurospora* does not depend on methylation. This conclusion is based on results of Southern blot experiments using pairs of methylation-sensitive restriction enzymes, using an inhibitor of methylation or a mutant defective in methylation. To distinguish whether quelling is due to transcriptional inactivation or a post-transcriptional process such as RNA turnover, an RNAse protection assay was done. A labelled RNA probe complimentary to the *al-1* gene was prepared and incubated with cellular RNA. After the unhybridized portion was removed by nuclease digestion, the size of the protected fragments was analyzed by gel electrophoresis. The amount of primary transcript (precursor mRNA) in quelled transformants was unchanged but the level of specific mRNA for the duplicated gene was reduced, leading to the conclusion that quelling is due to post-transcriptional gene silencing.

To understand the components of the machinery by which post-transcriptional silencing is brought about, a quelled strain (albino) was mutagenized and mutants (orange phenotype) were isolated that were impaired in quelling (Cogoni and Macino, 1997). By transformation of an albino-quelled strain with a plasmid (insertional mutagenesis), quelling deficient (*qde*) mutants were isolated that were orange in color. The rescued plasmid contained the putative *qde* gene whose sequence showed homology to RNA-dependent RNA polymerase. The experiments identified *qde* genes as a component of the silencing machinery. It is postulated that the *qde* product is RNA-dependent RNA polymerase that makes an antisense mRNA that causes the loss of transformed phenotype.

An interesting question arose whether the presence of transgene and endogenous gene in the same nucleus is required for silencing. A heterokaryon was constructed between quelled (albino phenotype) and wild strains (orange color) containing both *al-1* silenced and non-silenced nuclei. The white color of the heterokaryon demonstrated that quelling is dominant and the presence of transgene and endogenous gene in the same nucleus is not a prerequisite for silencing. Moreover, the use of heterokaryon demonstrated that silencing could occur through a diffusible trans-acting molecule (Figure 9.9). A heterokaryon constructed between a *qde* mutant that produced no transgenic sense RNA and a wild strain had an orange color, demonstrating that transgenic sense RNA is essential for silencing in heterokaryons. Presumably, the *qde* products and transgenic RNA interact to form a complex for degradation of endogenous mRNA and cause silencing.

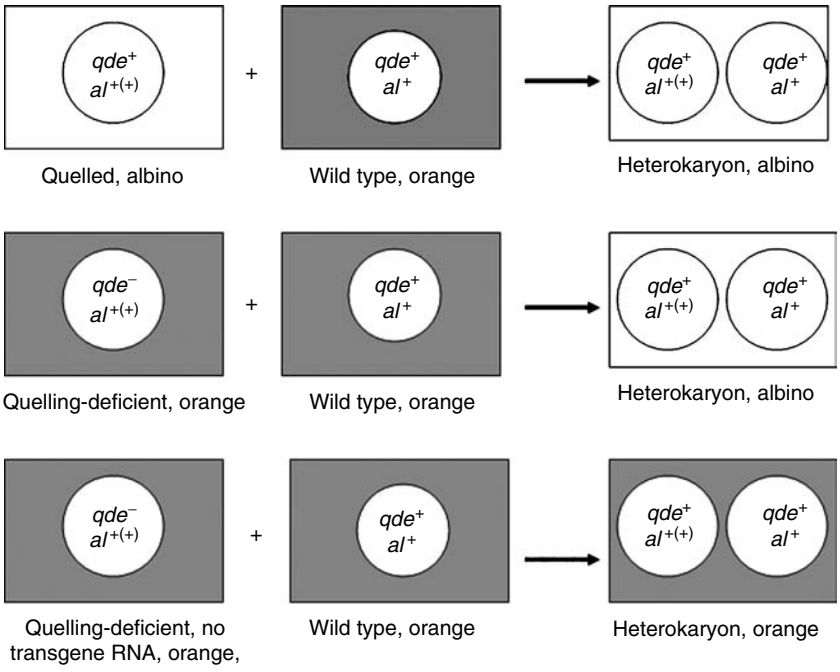

Figure 9.9 Diagram of quelling in heterokaryon. Gene introduced by transformation are shown in parentheses. Only one nucleus of each type is shown.

9.4.5 Internuclear Gene Silencing

A puzzling case of gene silencing was discovered in the potato pathogen *Phytophthora infestans* (Straminipila) that is maintained in the progeny nuclei even in the absence of the transgene (van West et al., 1999). The *inf-1* gene encodes a secretory protein called elicitin—a hydrophobin that induces necrosis in the plant, thereby restricting the spread of pathogenic mycelium. By transforming *P. infestans* with the plasmid-containing *inf-1* gene, mutants were produced that were silenced in the production of elicitin. The protoplasts of silenced and non-silenced strains were fused to obtain a heterokaryotic (silenced) strain that was resolved into homokaryotic component strains by nuclear separation using the uninucleate zoospores of this "fungus" (see Appendix for figure). The individual nuclear types multiplied mitotically in the regenerating mycelium. The homokaryotic strains produced from uninuclear zoospores were silenced, i.e., once gene silencing was induced, it was maintained in the homokaryotic strain even in the absence of the transgene. Though both internuclear gene silencing (IGS) and quelling are dominant, IGS differs from quelling in being infectious, i.e., transmitted from nuclei to nuclei, whereas quelling is not transmitted. Moreover, where the presence of transgene is essential for quelling, it is not required for IGS (Figure 9.10).

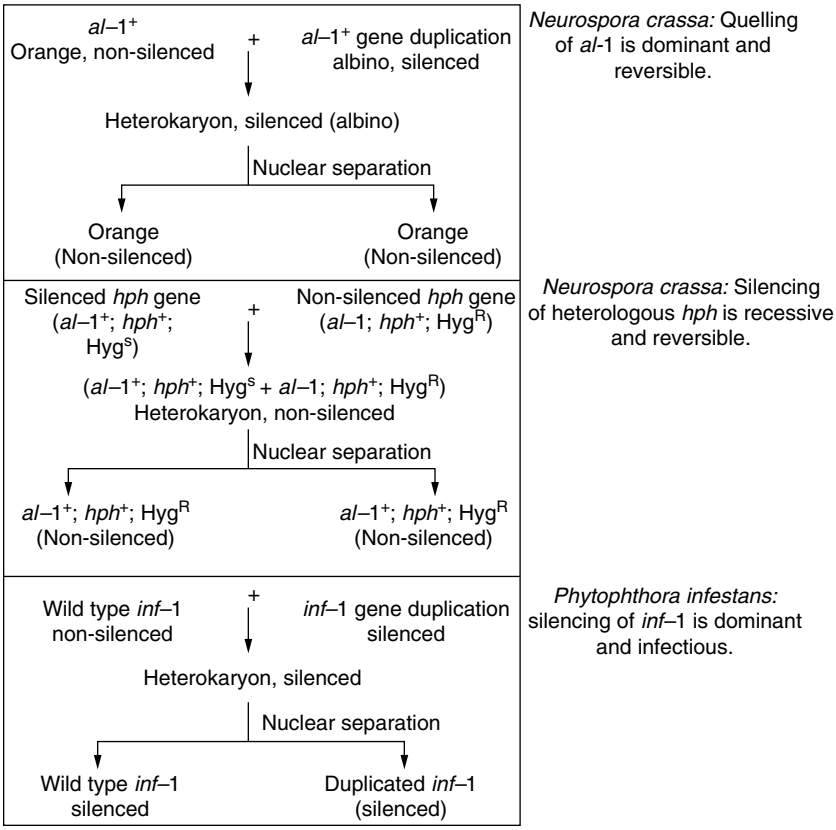

Figure 9.10 Transgene silencing in heterokaryons of *Neurospora crassa* and *Phytophthora infestans*. Adapted from Dev and Maheshwari (2003).

9.5 CONCLUDING REMARKS

DNA has been extracted from 18 million-year-old fossilized leaves dating to the Miocene period, suggesting that it is an inherently stable molecule. This presents the possibility that DNA molecules from dead organisms or viruses in the environment can recombine with nuclear DNA, causing changes in genome structure and function. Therefore, early in evolutionary history organisms must have developed defense mechanisms to detect sequence duplication and to protect nuclear DNA from unpredictable and unscheduled changes. The introduction of transgenes has led to the recognition that cells have multiple mechanisms that monitor the arrangement and content of the genome, detect sequence duplications and silence them in vegetative or sexual phases, or both. A great advantage offered by fungi is the feasibility of constructing heterokaryons. The experimental results with heterokaryons suggest one possible silencing mechanism—a diffusible transgene product, presumably aberrant RNA, is recognized by an RNA-dependent RNA polymerase, leading to the production of antisense RNA, formation of double stranded RNAs and RNA degradation.

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Part V

Adaptations

Chapter 10

Thermophilic Fungi: Eukaryotic Life at High Temperature

> It is of interest to study the systematics, distribution, and physiological adaptations of organisms which have been successful in colonizing high temperature environments in order to examine the limits to which evolution can be pushed. From an ecological point of view, high temperature environments usually have relatively simple species composition and short food chains, which make a study of productivity, trophodynamics, population fluctuation, and species interaction more simple. From the viewpoint of applied ecology, an understanding of the biology of high temperature habitats is essential if we are to predict and control the consequences of thermal pollution by various industrial sources.
>
> *Thomas D. Brock*

The vast majority of fungi best grow between 20 and 37°C. This temperature range is universally accepted as moderate. The vegetative mycelium of these fungi, called the *mesophilic fungi*, cannot survive prolonged exposure above 40°C. However, currently some 30 species of fungi are known which show optimum growth between 40 and 50°C, with a few species capable of growth up to 62°C. These species are grouped as *thermophilic fungi* with their minimum temperature of growth around 20 to 25°C. The temperature range for growth of either the thermophilic or the mesophilic fungi is about 32°C. This means that while the maximum temperature of growth of thermophilic fungi is extended, their minimum temperature of growth too has been raised.

While some species of bacteria and archaebacteria found in hot springs, solfataras or hydrothermal vents grow at temperatures between 80 to 113°C (Brock 1995; Blöchl et al., 1997), the thermophilic fungi are the only eukaryotes with the exception of the alga *Cyanidium caldarium* that thrive between 45 and 60°C—temperatures at which no plant or animal can survive for long (Figure 10.1). Thermophilic fungi are therefore valuable systems for investigations of genetic and biochemical mechanisms that allow higher organisms to adapt and deal with heat stress. They are arbitrarily distinguished from the thermotolerant fungi that have a minimum temperature of growth between 12 and 17°C and a maximum temperature of growth between 50 and 62°C. In general morphology and ultrastructural features, the thermophilic fungi are indistinguishable from the mesophilic or the thermotolerant fungi (e.g., *Aspergillus fumigatus*, which grows in a temperature range from 12 to about 55°C, http://helios.bto.ed.ac.uk/bto/microbes/thermo.htm).

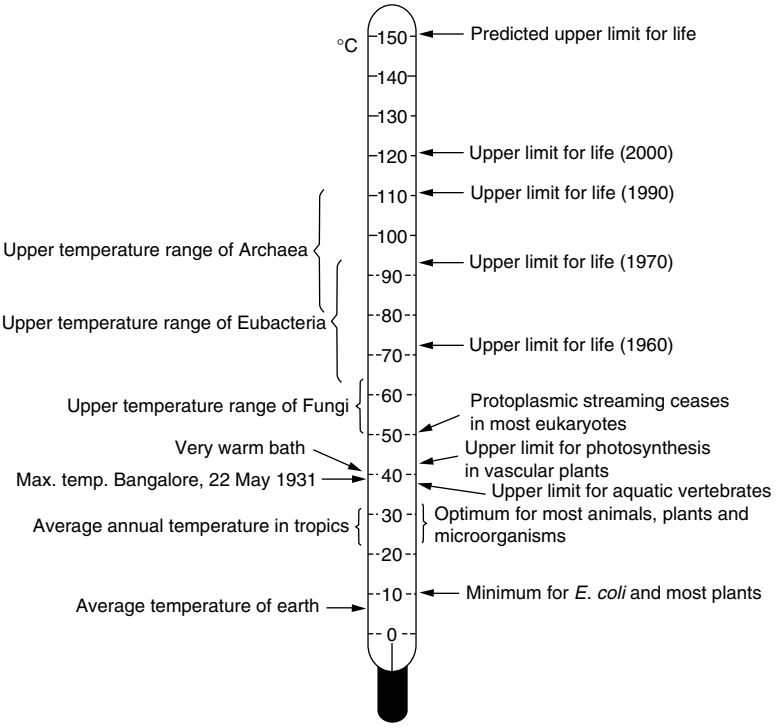

Figure 10.1 Thermometer of life (°C).

10.1 DISCOVERY

10.1.1 Self-Heating of Stored Agricultural Products

Thermophilic fungi were discovered as chance contaminants of bread or potato that had been inoculated with garden soil (see Cooney and Emerson, 1964; Maheshwari et al., 2000). Their habitats and growth conditions were discovered when Hugo Miehe (1907) of Germany was drawn to investigate the cause of self-heating and the spontaneous combustion of damp stacks of hay. He studied the role of microbial flora in thermogenesis. From the self-heating haystacks, Miehe isolated several microorganisms, including four species of thermophilic fungi: *Mucor pusillus* (renamed *Rhizomucor pusillus*, http://helios.bto.ed.ac.uk/bto/microbes/thermo.htm), *Thermomyces lanuginosus* (syn. *Humicola lanuginosa*, http://helios.bto.ed.ac.uk/bto/microbes/thermo.htm), *Thermoidium sulfureum* (renamed *Malbranchea cinnamomea*) and *Thermoascus aurantiacus* (http://helios.bto.ed.ac.uk/bto/microbes/thermo.htm). To assess their role in the self-heating of agricultural residues, Miehe inoculated moist hay and other plant materials kept inside thermal flasks with pure cultures of individual fungi. Whereas sterilized hay did not generate heat, that inoculated with the fungus did and the final temperature attained by the material showed a correlation with the maximum temperature of growth of the fungus used. Further, by controlled experiments, Miehe demonstrated that the naturally occurring microorganisms in moist haystacks or other plant materials caused its heating. Cooney and Emerson (1964) explained spontaneous combustion as follows: Initially, the heat produced from the

exothermic metabolic reactions in the mesophilic fungi raises the temperature of the compacted mass of vegetable matter to approximately 40°C, with this warm environment favoring the development of thermophilic fungi and actinomycetes present therein. The latter raise the temperature to about 60°C or higher, corresponding with their upper temperature limit of growth. Above 60°C, the mycelial growth of thermophilic fungi declines and they survive as heat-resistant spores, whereas the actinomycetes raise the temperature of the mass up to their maximum around 75°C. Beyond this temperature, autocatalytic chemical reactions are triggered which ignite the haystack. Figure 10.2 is a photograph of the spontaneous combustion of a large pile of sugar cane bagasse near a sugar manufacturing factory. The discovery of thermophilic fungi provided a link in the puzzle of spontaneous combustion of stored agricultural products.

Figure 10.2 Spontaneous combustion of a heap of sugar cane bagasse near a sugar factory in India.

10.1.2 Guayule Rets

During the years of the Second World War, the need for finding alternate sources of rubber led to studies of the latex-bearing guayule plant *Parthenium argentatum* as a potential source. The extractability and quality of rubber improved if, before milling, this shrub was chopped and stored in a mass ("rets") that self-heated to temperatures between 65 and 70°C. From the guayule rets, Paul J. Allen and Ralph Emerson (1949) isolated some ten species of thermophilic fungi with temperature limits up to 60°C and all species capable of decomposing resin. The improvement from retting resulted primarily from the reduction in the amount of contaminating resin in the latex by a thermophilic microflora. Allen and Emerson found that for the optimal development of thermophilic fungi in the guayule rets, its moisture content, porosity and size were crucial factors for the build up of microbial protoplasm, for their aerobic respiration and for insulation against the loss of heat produced by their metabolism.

10.1.3 Composts

Compost is decomposed plant debris prepared by gathering refuse material in a heap to hasten the decay of the material and reduce its bulk. The heat produced in the heaped mass of garbage, plant residues, herbivore dung and kitchen and municipal waste kills pests, mesophilic microorganisms and drives off toxic ammonia. The process of production of organic manure by composting is an unwitting exploitation of thermophilic microorganisms. A similar exploitation of thermophilic fungi is in the preparation of substrate for the cultivation of the edible mushroom *Agaricus bisporus*. A mixture of herbivore dung and straw is composted to give the material a physical texture that favors the growth of mushroom mycelium. Thermophilic fungi, in particular *Scytalidium thermophilum* (syn. *Torula thermophila*, *Humicola grisea* var. *thermoidea*, *Humicola insolens*), play a dominant role in the preparation of mushroom compost (Straatsma and Samson, 1993). The majority of the about 30 currently known species of thermophilic fungi were originally isolated from composts of various types.

Since composts are man-made environments, it is arguable whether they can be considered as natural habitats where thermophilic fungi evolved. Cooney and Emerson (1964) suggested that the nests of the incubator birds (mallee fowl), a species existing for 50 to 60 million years, as a possible natural habitat of thermophilic fungi. These are large-sized birds found in Australia and islands of the southwestern Pacific that gather forest litter and soil and construct large mounds inside which eggs are laid for incubation (http://www.abc.net.au/science/scribblygum/October2000/default.htm). The interior temperature ranges from 33 to 50°C for several months. Perhaps such warm, humid and aerobic environments were sites where thermophilic fungi evolved from the mesophilic forms. The limited species diversity of thermophilic fungi suggests they are of relatively recent origin.

10.2 DISTRIBUTION IN SOIL

Thermophilic fungi have been isolated from almost any soil, even in the temperate zones, prompting the remark "the ubiquitous distribution of organisms, whose minimal temperature for growth exceeds the temperature obtainable in the natural environment from whence they were isolated, still stands as a 'perfect crime' story in the library of biological

systems" (Tendler et al., 1967). Whether their presence in soil is because of their growth therein or a consequence of dissemination of their spores from compost heaps that occur world-wide has not been easy to resolve since the opaqueness of soil precludes microscopic examination of fungal growth. Therefore, several indirect approaches have been taken to assess soil as a habitat of thermophilic fungi. Eggins et al. (1972) used a soil immersion tube (Figure 10.3) for discriminating the mycelial form from the dormant spores. A cellulose paper strip (a source of carbon) was enclosed inside a screen of glass fiber and placed in soil so that only the active mycelium could penetrate through the screen and colonize the substrate. The tubes were removed from the soil at intervals and the paper strips plated on cellulose agar to determine if these were invaded by mycelium from the soil. By this method the thermophilic fungi *Humicola grisea* and *Sporotrichum thermophile* were detected in the sun-heated soil. It would appear that this device had the potential of picking out active mycelium but Tansey and Jack (1976) pointed out that an incorrect impression of growth of fungi can occur if spores were passively carried onto the test substrate by soil arthropods or by capillary action. Therefore, these authors studied the

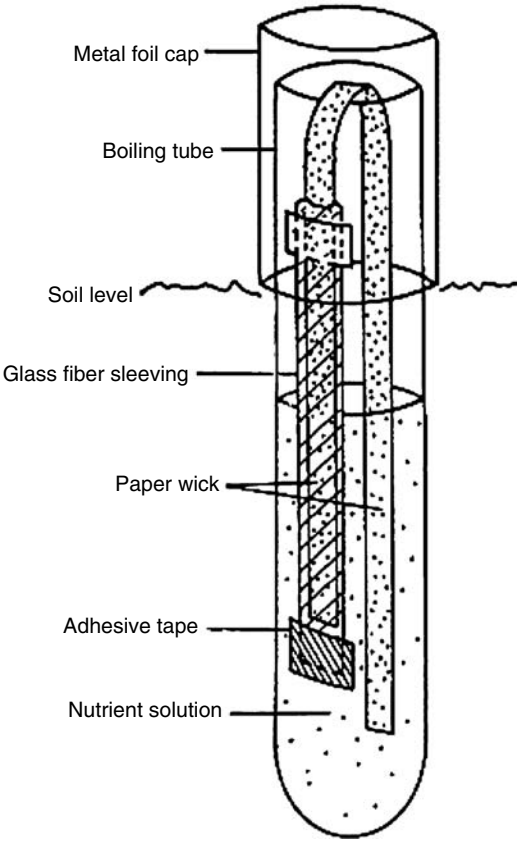

Figure 10.3 Diagram of soil immersion tube. (From Eggins et al. (1972). With permission from Elsevier.)

development of spores of individual species of thermophilic fungi in petri dishes containing pure cultures that were buried in field soil in the US state of Indiana. All species tested germinated and developed sporulating colonies in the buried plates, leading the authors to the view that the extent and duration of elevated temperature reached in the sun-heated soil are sufficient for thermophilic fungi to grow therein. However, this extrapolation from pure cultures in nutrient media in buried petri dishes is also doubtful since the propagules of thermophilic fungi co-exist with microbes, mesophilic fungi and microfauna that are potential competitors.

Rajasekaran and Maheshwari (1993) attempted to forecast the potential of thermophilic fungi to grow in soil based on competitive growth in mixed cultures under a fluctuating temperature regime. Incubation of soil plates in a programmed incubator at temperature regimes from 24 to 48°C, 32 to 48°C and 36 to 48°C (Figure 10.4) yielded mesophilic fungi or a thermotolerant fungus, *Aspergillus fumigatus*. At a temperature regime from 36 to 48°C, the predominant fungi that developed in soil plates were still mesophilic types with occasional colonies of the thermophilic fungus *Humicola grisea* var. *thermoidea*. Thermophilic fungi developed only when the incubation temperature fluctuated to a small extent between 40 and 48°C, about the summed average optimum temperature (46°C) of common thermophilic fungi. The results suggested that high temperatures are necessary for thermophilic fungi to compete with the numerically larger mesophilic population in soil. Several workers have isolated thermophilic fungi from desert soils that heats up to 50°C or more with the implied conclusion that desert soil is a natural habitat of thermophilic fungi. However, since liquid water is essential for growth, it is unlikely that the presence of spores of thermophilic fungi in dry soils is a consequence of their growth *in situ*.

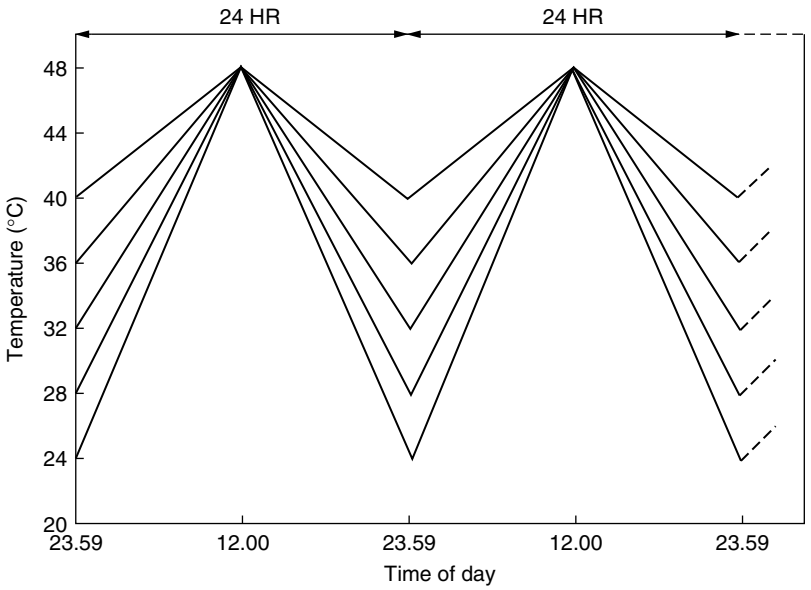

Figure 10.4 Diagram of five diurnally fluctuating temperature regimes used to study competitive growth of a mixed soil fungal flora in soil plates in nutrient medium. (From Rajasekaran and Maheshwari (1993).)

In yet another approach, Rajasekaran and Maheshwari (1993) used immunofluorescence microscopy to detect the growth of a ubiquitous thermophilic fungus in soil. Spores of *Thermomyces lanuginosus* were adhered to glass cover slips and buried in field soil where moisture levels seemed favorable for microbial growth. The soil temperature varied between 22 and 28°C during the time of the experiment. After three weeks, the cover slips supporting the spores were retrieved and stained with fluorescein isothiocyanate (FITC) conjugated antibody prepared using germinating spores of the test fungus. Only dormant spores were seen, although spores similarly placed in petri dishes in unsterilized soil at 50°C in a laboratory incubator had germinated. These results did not support the view that soil is a "natural habitat" of thermophilic fungi.

Various air-sampling devices have captured spores of thermophilic fungi from air even in the temperate regions. The aerial dissemination of spores from world-wide composts of various types and their fall-out from air can explain the widespread occurrence of thermophilic propagules in soil. The concentration of spores of thermophilic fungi per gram material is approximately 10^6 times higher in composts than in soils (see Maheshwari, 1997). Thermophilic fungi are primarily compost fungi.

10.3 PHYSIOLOGY

10.3.1 Cultivation

The early workers reported that successful cultivation of thermophilic fungi requires supplementation of culture medium with a decoction of hay, casamino acids, peptone, yeast extract or a tricarboxylic acid cycle intermediate (for example, succinic acid), leading them to the view that thermal adaptation is associated with special nutritional requirements. However, some species were subsequently grown in synthetic minimal media (Gupta and Maheshwari, 1985) containing a carbon source (glucose), a nitrogen source (ammonium phosphate, urea or asparagine, a source of sulfur (magnesium sulfate), a source of phosphorus and buffering anions (phosphate) and trace elements. The pH optima of the thermophilic fungi studied are between 7.0 and 8.0, close to the pH level of composts. Control of the pH of growth media is an important factor in the cultivation of thermophilic organisms because at elevated temperatures carbon dioxide becomes unavailable due to its reduced solubility, more so if acidic conditions develop. If, for example, ammonium sulfate is used in culture media as a source of nitrogen, the absorption of ammonium ions and the counter transport of H^+ from the cells results in the rapid acidification of the medium and the growth stops. The acidic conditions that develop reduce the solubility of CO_2 required for anaplerotic reactions, for example, the reaction catalyzed by the enzyme pyruvate carboxylase:

$$\text{pyruvate} + CO_2 + \text{ATP} \rightarrow \text{oxaloacetate} + \text{ADP} + P_i$$

This enzyme functions to replenish the intermediates of the tricarboxylic acid cycle as they are used up in generating energy and for biosynthesis. Thus, for growth to continue the medium needs to be supplemented with a tricarboxylic acid cycle intermediate such as succinic acid. Increasing the buffering capacity of the medium, or automatic pH control by the addition of alkali in an instrumented fermentor, are the methods commonly used for pH control. A convenient practice is to replace the inorganic nitrogen source with an organic nitrogen source, for example, L-asparagine.

10.3.2 Metabolic Rate

In 1920, Kurt Noack of Germany compared the metabolic rates of thermophilic and mesophilic fungi at different temperatures. Using the volume of carbon dioxide evolved over time as a measure of the metabolic rate, he compared *Thermoascus aurantiacus* (a thermophilic fungus) with *Penicillium glaucum* (a mesophilic fungus), both grown in identical media. The quantity of carbon dioxide released by the mesophilic fungus in 24 hours was equivalent to 67% of its dry weight at 15°C and 133% at 25°C. Noack reasoned that if this fungus could grow at 45°C, the extrapolated value of carbon dioxide according to the van't Hoff rule would be 532%. However, the actual value for the thermophilic fungus used at 45°C was 310%. From this, Noack inferred that at a given temperature the metabolism of a thermophilic fungus is actually slower than what is expected based on the van't Hoff rule.

Subsequent measurements of oxygen uptake of mycelial suspensions by the Warburg method show that at their respective temperature optima, thermophilic fungi have a respiratory rate comparable to the mesophilic fungi (Prasad et al., 1979; Rajasekaran and Maheshwari, 1993). An unexpected observation was that the respiratory rate of mesophilic fungi is temperature-compensated over a broad range of temperatures (Figure 10.5). All major chemical components—proteins, lipids and nucleic acids—have their structural and functional properties altered by changes in temperature. How the mesophilic fungi achieve the observed metabolic homeostasis at a broad range of temperatures is not known. The adaptive modification of their basic biochemical structures and functions to exploit the natural environments continues to be the focus of investigations on biochemical adaptation.

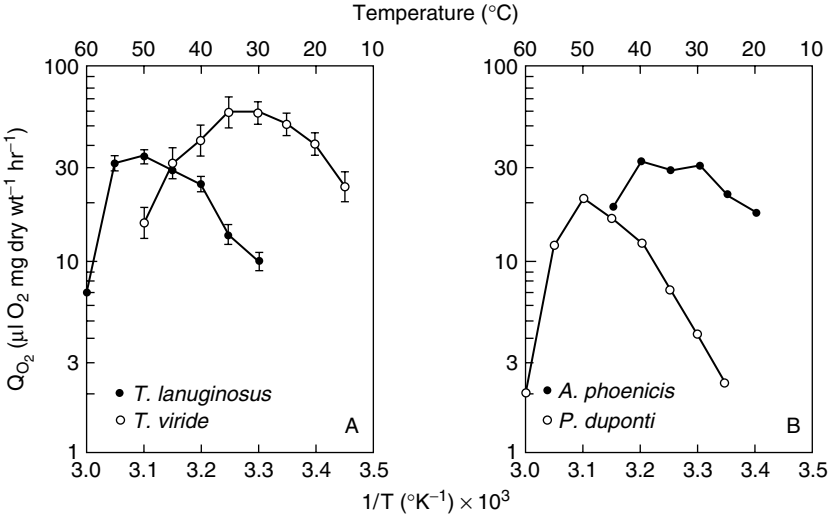

Figure 10.5 Respiration of mycelia of thermophilic (*Thermomyces lanuginosus*, *Penicillium duponti*) and mesophilic (*Trichoderma viride*, *Aspergillus phoenicis*) fungi measured by Warburg manometric method. The data is given as Arrhenius plots of the logarithm of respiratory rate (Q_{O2}) of shaker-grown mycelia against reciprocal of absolute temperature. (From Rajasekaran and Maheshwari (1993).)

10.3.3 Efficiency of Growth

Two parameters are commonly used to compare the growth efficiency of different fungi. One is the exponential growth rate (μ), determined from the exponential portion of semi-logarithmic plots of the growth curve:

$$\mu = 2.303\left(\frac{\log x_2 - \log x_1}{t_2 - t_1}\right) \text{h}^{-1}$$

where x_1 and x_2 are the dry weight in milligrams at time t_1 and t_2, respectively. The other parameter is the molar growth yield (Y_G), determined as yield (dry weight of mycelium) per mole of glucose utilized. Shaker-grown mycelia of some thermophilic fungi yield a homogeneous mycelial suspension that is amenable to pipeting, allowing sampling and quantitative measurements of the above parameters. For thermophilic and mesophilic fungi, the specific growth rates were 0.23 h^{-1} vs. 0.16 to 0.37 h^{-1}, respectively, and the average Y_G value was 86 vs. 88 g/mole (Maheshwari et al., 2000). On average, both mesophilic and thermophilic fungi convert approximately 55% of sugar for the synthesis of biomass and 45% for metabolism. This situation is similar to that in thermophilic bacteria.

10.3.4 Carbon Sources in Environment

As the temperature begins to rise in a compost heap, the mesophilic microflora is succeeded by thermophilic microflora (Hedger and Hudson, 1974). Therefore, the availability of soluble carbon sources (sugars, amino acids and organic acids) will decrease and the carbon source available to thermophilic fungi will mainly be the polysaccharide constituents of the biomass, chiefly cellulose and hemicelluloses. Not surprisingly, thermophilic fungi are therefore especially well adapted for polysaccharide utilization. The growth rate of the thermophilic fungus *Sporotrichum thermophile* on cellulose (paper) is similar to that on glucose (Bhat and Maheshwari, 1987). *Chaetomium thermophile* and *Humicola insolens* grew better on xylan than on simple sugars (Chang, 1967).

10.3.4.1 Mixed Substrate Utilization

In composting plant material, the hydrolysis of polysaccharide constituents by the secreted enzymes is expected to produce a mixture of sugars in the growth environment of thermophilic fungi. One of the adaptive strategies for their growth could be the simultaneous utilization of a mixture of sugars. To test this, the thermophilic fungi *Thermomyces lanuginosus* and *Penicillium duponti* were grown in a mixture of glucose and sucrose in liquid media (Maheshwari and Balasubramanyam, 1988). Both fungi concurrently utilized glucose and sucrose at 50°C, with sucrose utilized faster than glucose (Figure 10.6). This is quite the opposite of the phenomenon of diauxy observed in bacteria that utilize one carbon source at a time, e.g., glucose is utilized before lactose in a mixture of the two sugars. The simultaneous utilization of sucrose in the presence of glucose occurred because invertase is insensitive to catabolite repression by glucose and because the activity of the glucose uptake system is repressed by sucrose.

10.3.5 Transport of Nutrients

Nutrients from the environment are transported inside the cell by specific carrier proteins in the plasma membrane. One reason why thermophilic fungi fail to grow at ordinary

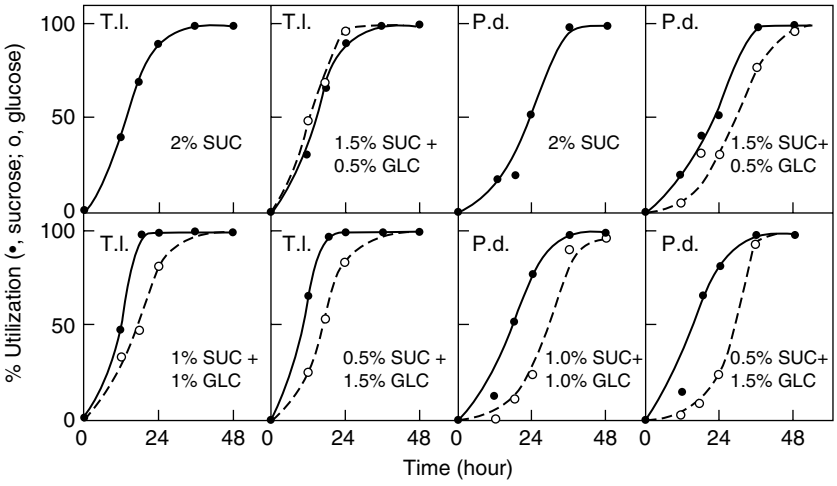

Figure 10.6 Simultaneous utilization of glucose and sucrose by two thermophilic fungi in shake flask culture. T.l., *Thermomyces lanuginosus*; P.d., *Penicillium duponti*. The fungus was grown in a liquid medium containing the indicated concentrations of sugars. Growth and utilization of sugars were monitored at indicated time. (from Maheshwari and Balasubramanyam (1988). American Society of Microbiologists. With permission.)

temperatures could be because their transporter proteins are transformed into a rigid conformation affecting the binding and release of ions and nutrients and therefore functioning very poorly. A future direction of the work will be the cloning of transporters from mesophilic and thermophilic fungi, their reconstitution in lipid vesicles (liposomes) and comparative study of the kinetics of transport at a range of temperature.

10.3.6 Protein Turnover

An early hypothesis put forward to explain thermophily in bacteria proposed that growth at high temperatures occurs because the denatured cellular proteins are quickly replaced by resynthesis. Two different groups compared the rate of protein breakdown in thermophilic and mesophilic fungi by feeding mycelia with radioactively labeled amino acid and monitoring the radioactivity in mycelial proteins after transferring mycelia to non-radioactive media (Miller et al., 1974; Rajasekaran and Maheshwari, 1990). Although both groups measured only protein breakdown, similar results are expected had protein turnover been measured. The rapid protein turnover hypothesis is not yet substantiated but since at their respective temperature optima both mesophilic and thermophilic fungi produce comparable amounts of mycelium, it is unlikely that this hypothesis is valid generally because energy expended in increased protein turnover in thermophilic fungi would be expected to affect their yield, which does not happen. However, as shall be noted in Section 10.5.2, certain enzymes could have a rapid turnover rate.

10.4 SECRETORY ENZYMES

There is a great practical interest in thermophilic fungi because of the prospects of finding enzyme variants with high temperature optima and long "shelf life," desirable properties

for enzymes used in various industries. Chiefly, the extracellular (secreted) enzymes have been studied as the mycelium is easily removed by the filtration method and large amounts of culture filtrates obtained as starting material for enzyme purification. The extracellular enzymes of thermophilic fungi have molecular masses ranging from 20 to 60 kDa and are thermostable (Maheshwari et al., 2000). Examples of a few extracellular enzymes purified from cultures of thermophilic fungi are given below.

10.4.1 Proteases

Proteases act on proteins or polypeptides. Historically, attention on thermophilic fungi was advanced by the finding of a strain of *Mucor* that produced milk-curdling activity that could substitute for chymosin (rennin), required in the manufacture of cheese. Previously, rennin was obtained from the stomach of suckling calves. The *Mucor* acid protease, induced in media containing wheat bran, had maximal activity at a pH of 3.7 and was stable at 55°C. The *Mucor* rennin gene was expressed in yeast that secreted the foreign (heterologous) protein at concentrations exceeding 150 mg/l. A recombinant mesophilic fungus, *Aspergillus oryzae*, produced *Mucor* acid protease in excess of 3 grams per liter of medium. Among the varied applications of proteases are the tenderizing of meat, manufacture of cheese, a component of detergents for the removal of proteinaceous dirt from fabric and for dehairing of leather (Johri et al., 1999).

10.4.2 Lipases

Lipases hydrolyze triacylglycerols. Lipases, stable at 65°C and at a pH between 10 and 11, have application as ingredients of detergents for removing oil stains by hot water machne wash. Strains of *Thermomyces lanuginosus* (equiv. *Humicola lanuginosa*) secrete appreciable quantities of lipase when grown in media containing animal or vegetable oils as an inducer. The lipase gene from *T. lanuginosus* and *Rhizomucor miehei* (syn. *Mucor miehei*) were cloned and expressed in a heterologous mesophilic fungus, *Aspergillus oryzae*. The thermophilic *R. miehei* lipase was the first lipase whose three-dimensional structure was deduced by x-ray analysis. The catalytic site of the lipase protein is covered by a short α-helical loop that acts as a "lid" that moves when the enzyme is adsorbed onto the oil-water interface, allowing the access of the substrate to the active site.

10.4.3 Amylases

Amylases are a group of enzymes that hydrolyze starch, forming oligosaccharides, maltose or glucose. Amylases have applications in the industrial scale conversion of starch into glucose. For this, heat stable amylases are preferred since the saccharification reaction for the manufacture of glucose syrups can be done in large reactors between 70 and 80°C with a minimum threat of contamination by common microbes in the environment.

Starch is hydrolyzed by two main types of enzymes: an endo-acting α-amylase that produces maltooligosaccharides and an exo-acting glucoamylase that produces chiefly glucose. *T. lanuginosus* produces both enzymes that are fully stable at 50°C, though inactivated at 70°C (Mishra and Maheshwari, 1996). In the presence of calcium, α-amylase is nearly eight times more stable at 65°C. The α-amylase of *T. lanuginosus* is a dimer, which upon heating to the boiling temperature of water undergoes a structural reorganization and is progressively converted to an inactive trimeric species by the self-association of subunits.

The starch-degrading enzymes of *T. lanuginosus* are the most thermostable enzymes among fungal sources. The high purity of products (glucose and maltose) and their thermostability suggests the potential usefulness of *T. lanuginosus* glucoamylase and α-amylase in the enzymatic saccharification of starch.

10.4.4 Cellulases

Cellulases refers to a group of enzymes (endoglucanase, exoglucanase and β-glucosidase) that act together to solubilize cellulose. Cellulase enzymes are used in detergent formulations to remove unwanted pill-like balls of fuzz that form on clothes due to repeated washing and wearing. The endoglucanases (molecular weight ranging from 30 to 100 kDa) are thermostable and active between 55 and 80°C at a pH between 5.0 and 5.5. The exoglucanases (molecular weightt between 40 and 70 kDa) are thermostable glycoproteins that are optimally active between 55 and 75°C. The molecular characteristics of β-glycosidase are variable, with a molecular weight ranging from 45 to 250 kDa and carbohydrate content ranging from 9 to 50%. Except for their thermostability, the molecular characteristics of cellulase components of thermophilic fungi are quite similar to those from mesophilic fungi. *T. aurantiacus* grown on paper and *H. insolens* grown on wheat bran are exceptionally good producers of cellulase enzymes.

10.4.5 Xylanases

Several thermophilic fungi are exceptionally high producers of xylanases with the application in the bio-bleaching of wood pulp for paper manufacture. They are generally single-chain glycoproteins, ranging from 6 to 80 kDa, active between a pH of 4.5 to 6.5, and at temperatures between 55 to 65°C. Xylanases are co-induced with cellulases by natural substrates or inferior quality filter-paper containing hemicelluloses. *Thermoascus aurantiacus* and *Paecilomyces varioti* are exceptionally high producers of xylanase—the enzyme simply crystallized out in concentrated protein solution facilitating structural analysis by x-ray (Maheshwari et al., 2000). The thermostability of xylanases from thermophiles is postulated to be due to an extra disulfide bond and the preponderance of salt bridges holding the secondary structure of protein.

10.5 CELL-ASSOCIATED ENZYMES

Because of the general difficulty in disrupting mycelium and extracting cellular protein from mycelia, few attempts have been made to compare functional or physical-chemical properties of homologous cytosolic enzymes from mesophilic and thermophilic fungi. Here two examples of glycosidases are given that show contrasting behavior (Figure 10.7).

10.5.1 Trehalase

The substrate of the enzyme trehalase is trehalose, commonly called mushroom or fungal sugar. It is a disaccharide composed of two glucose units joined by an α,1→1 glycosidic bond (Figure 10.8). Remarkable effects of trehalose on the protection of membranes and thermostabilization of enzymes has been found (Crowe et al., 1992). Bharadwaj and Maheshwari (1999) chose the enzyme trehalase (which hydrolyzes trehalose) for a comparative investigation in a thermophilic and a mesophilic fungus. *Thermomyces lanuginosus* grows optimally between 50 and 55°C, whereas *Neurospora crassa* grows optimally

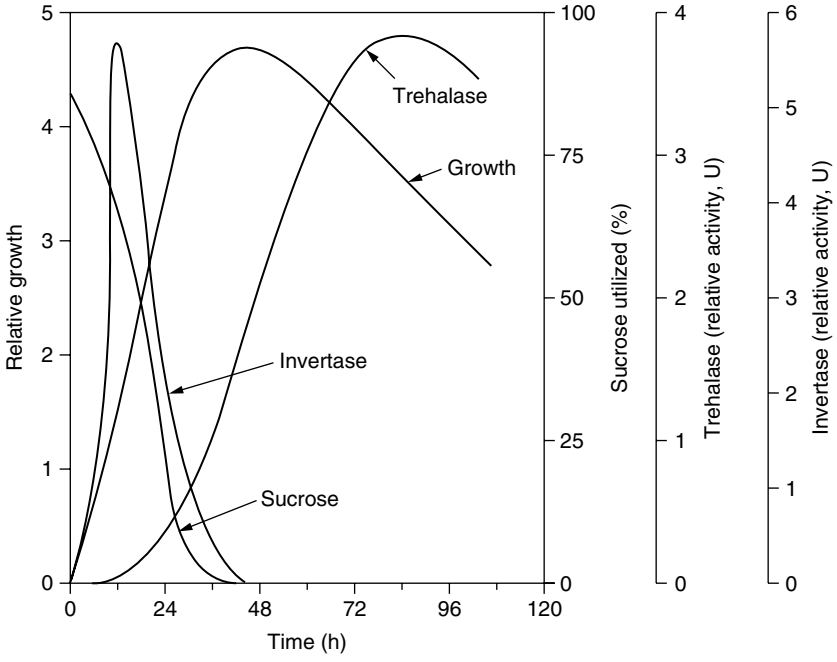

Figure 10.7 Contrasting pattern of development of invertase and trehalase activities in *Thermomyces lanuginosus*. Fungus was grown in a liquid medium containing sucrose as the carbon source. (From Chaudhuri et al. (1999). With permission from Elsevier.)

between 30 and 35°C. The following conclusions can be made from a study of trehalase from these two fungi. (i) Although the upper temperature limit for growth of *N. crassa* and *T. lanuginosus* are quite different (40 vs. 60°C), trehalase from both fungi had similar temperature optima for activity (50°C), showing that the temperature optimum of an enzyme may not be not related to the growth temperature of the organisms. (ii) Trehalase of *T. lanuginosus* and *N. crassa* are structurally very different proteins (monomer vs. tetramer). (iii) At 50°C, the $t_{1/2}$ of trehalases is in excess of 6 h. Trehalase from both

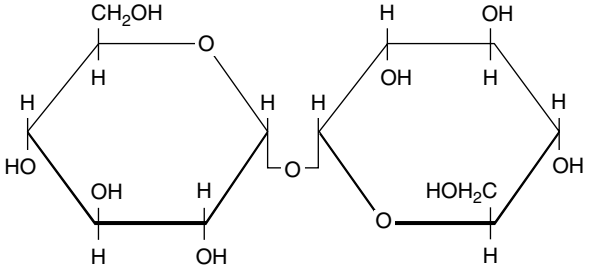

Figure 10.8 Trehalose. A non-reducing disaccharide composed of two glucose molecules joined by (α1→1) glycosidic bond.

sources was about equally stable. (iv) The k_{cat}/K_m of *T. lanuginosus* trehalase is one order of magnitude lower than that of the *N. crassa* enzyme—the enzyme from the mesophilic fungus is a better catalyst than from the thermophilic fungus.

10.5.2 Invertase

Invertase, an enzyme from yeast studied extensively that played an important role in the development of biochemistry, is synthesized constitutively in the mesophilic fungi, wherein its activity increases with an increase in growth (biomass). By contrast, it is an inducible enzyme in the two thermophilic fungi studied, i.e., it is produced only in response to the availability of sucrose in the growth medium (Table 10.1). Strangely, the induced enzyme activity begins to diminish before any substantial quantity of sucrose is utilized or an appreciable amount of biomass is formed (Chaudhuri et al., 1999). Paradoxically, despite this pattern of development of invertase, the final mycelial yields are not affected. An investigation of this unusual pattern of development of invertase activity in a thermophilic fungus, *T. lanuginosus*, has given some insight into various strategies in the biochemical adaptation of thermophilic fungi.

It seems likely that invertase in thermophilic fungi is localized in the hyphal tip. In the early stages of growth the number of tips per unit mass of mycelium is maximal and, correspondingly, the invertase activity is maximal. At later times, the number of tips per unit mass of mycelium decreases as the increase in mass is contributed mainly by cell elongation and cell wall thickening. Consequently, invertase-specific activity shows an apparent decline with growth. In contrast, in the mesophilic fungus *N. crassa*, invertase is distributed all along the hypha, an unquantitated amount being bound to the wall. Consequently, its specific activity steadily increases in proportion with the increase in growth measured as an increase in biomass.

Section 10.2 referred to an early finding of incongruity in the metabolic rate of thermophilic fungi and the van't Hoff rule (Noack, 1920). The invertase example hints on the nature of molecular evolution—not all proteins evolved thermostability at the

Table 10.1 Distinctive Properties of Invertase of a Thermophilic and a Mesophilic Fungus

Property	*Thermomyces lanuginosus* (Thermophilic)	*Neurospora crassa* (Mesophilic)
Synthesis	Induced by substrates of enzyme (β-fructofuranosides)	Constitutive, synthesized irrespective of carbon source in medium
Distribution	Hyphal tip	Uniformly present along hypha
Synthesis	Induced by substrates (β-fructofuranosides)	Constitutive (synthesized irrespective of carbon source in medium)
Relationship to growth	Activity inversely related to biomass	Activity parallels increase in biomass
Glucose repression	Not repressed	Repressed
Stability in cell-free extracts at 0°C	Unstable	Stable for more than one month
Effect of thiol compounds	Activated and stabilized	No effect

expense of a reduction in catalytic function. For these molecules, the cell must constantly expend energy on rapid resynthesis. This may explain the apparent incongruity of the respiratory rate with the van't Hoff rule. Interestingly, despite possessing unstable invertase and sucrose transporter, the growth rate and final yield in sucrose media is optimized in a fungus possessing unstable invertase and sucrose transporter. To offset this disadvantage, invertase and sucrose transporter are localized in the hyphal tip, which has a reducing environment and thereby maintains catalytically important sulfhydryl group(s) in protein molecules in the reduced state for enzyme function (Chaudhuri and Maheshwari, 1996).

10.6 CONCLUDING REMARKS

Although widespread in terrestrial habitats, thermophilic fungi remain underexplored. Because they occur in habitats that are heterogeneous in temperature, the types and concentration of nutrients, competing species and other variables, they probably also adapted to factors other than a high temperature. They can be grown in minimal media with metabolic rates and growth yields comparable to those of mesophilic fungi. Studies of their growth kinetics, respiration, mixed-substrate utilization, nutrient uptake and protein breakdown rate have provided some basic information not only on thermophilic fungi but also on fungi in general. Thermophilic fungi have a powerful ability to degrade the polysaccharide constituents of biomass. The properties of their enzymes show differences not only among species but also among strains of the same species. Their extracellular enzymes display temperature optima for activity that is close to or above the optimum temperature for the growth of the organism and, in general, are more stable than those of mesophilic fungi. Genes of thermophilic fungi encoding lipase, protease, xylanase and cellulose have been cloned and overexpressed in heterologous fungi, and pure crystalline proteins have been obtained for elucidation of the mechanism of their intrinsic thermostability and catalysis. By contrast, the thermal stability of the few intracellular enzymes that have been purified is comparable to or, in some cases, lower than that of enzymes from the mesophilic fungi. The gain of thermostability in certain intracellular proteins may not be possible without a concomitant loss of catalytic activity, as shown by the example of invertase. In the thermophilic fungi, this enzyme is exceptionally unstable, cautioning against the generalization that thermophily in fungi is due to the thermostability of proteins. There is no single adaptive strategy in fungi inhabiting a hot environment; rather, a combination of mechanisms allow a thermophilic fungus to adapt to a hot environment: the intrinsic thermostability of macromolecules, interaction of proteins with ions and other cellular proteins including chaperonin molecules, self-aggregation, and possibly covalent or noncovalent interactions with the cell wall, the placement of unstable, inducible enzymes in the most strategic location of the hypha and their resynthesis.

Currently, enzymes from hyperthermophilic archaea that grow at temperatures beyond 80°C are being sought for applications in biotechnology. However, since flexibility of protein conformation is essential for catalysis, the enzymes from the hyperthermophiles will have optimal conformational flexibility at the temperatures for which they are adapted to grow but could become too rigid and have low catalytic rates at temperatures that range from 50 to 65°C. Therefore, in most situations, enzymes from thermophilic fungi may be better suited in biotechnology than enzymes from hyperthermophilic archaea.

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Chapter 11

Photoresponses and Circadian Rhythm

Fungi live in terrestrial habitats. However, because fungi are non-green and their mycelium is generally hidden in substratum, the tendency is to disregard that light has any effect on them. Although generalizations cannot be made, the examples below will show the influence of light on fungal development. The genome analysis of the fungus *Neurospora crassa* led to unexpected identification of homologues of plant photoresponse proteins such as phytochromes and cryptochrome, sparking interest in the role of light in the development of fungi.

11.1 TYPES OF PHOTORESPONSES IN FUNGI

11.1.1 Pigmentation

A century ago, F.A.F.C. Went (father of the plant physiologist F.W. Went, discoverer of the plant growth hormone auxin) was stationed at the Bogor Botanic Gardens on the island of Java in Indonesia and noted that *Oidium aurantiacus* growing on bread developed an orange color in the daylight but remained white when grown in darkness. (This fungus was later renamed *Neurospora sitophila*.) To determine the active wavelength of light that induced pigmentation in this fungus, Went used a double-walled glass jar filled with different colored salt solutions that absorbed light of different wavelengths and acted as a light filter. He discovered that blue light was the active region of the spectrum that induced pigmentation. In *Neurospora*, the formation of conidia and protoperithecia and the phototropism of the perithecial neck are also blue-light responses.

11.1.2 Growth Rhythms

Several fungi growing on solid media develop a series of regularly spaced alternating thick and thin mycelium density, or alternating zones of sporulating and nonsporulating hyphae, called *zonations* (Jerebzoff, 1965). For example, cultures of *Sclerotinia fruticola* show zonations of decreasing intensity every 24 hours after dark-grown cultures are exposed to light (Figure 11.1). In dark-grown cultures of *Fusarium discolor* var. *sulfureum*, *Trichothecium roseum* and *Verticillium lateritium* (Fungi Anamorphici), daily illumination of 1000 to 3000 lux for a few seconds suffices to induce zonations. The rhythmic zonations can continue for some time after the fungus is transferred to complete darkness and is almost certainly

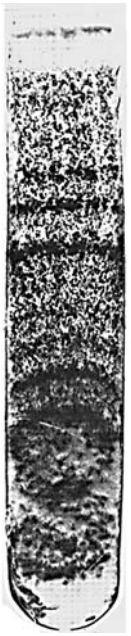

Figure 11.1 Growth rhythms in *Sclerotinia fruticola*. Culture maintained in dark for two days received a first photoperiod of 12 hours at 250 lux, and a second after five days. After each stimulus, four zonations of decreasing intensity occurred, one every 24 hours. (From Jerebjoff (1965). With permission of Elsevier.)

due to the diurnal periodicity of alternating light and darkness. This rhythmic growth pattern is a manifestation of an endogenous clock—they have a period of approximately 24 hours and are independent of temperature. In some, fungi zonation is stimulated by the supplementation of growth media by factors present in yeast, malt or potato extract (Jerebzoff, 1965), suggesting they need excitation by certain chemical factors for the manifestation of rhythms. The endogenous rhythms persist for a long time and continues without attenuating for three weeks in *Sclerotinia fruticola* (Ascomycotina), one month in *Aspergillus ochraceus* and *A. niger* and 70 days in *Alternaria tenuis* (Fungi Anamorphici).

11.1.3 Reproduction

More striking is the effect of light on reproduction in fungi. Colonies exposed to diurnal periodicity exhibit alternating zones of sporulating and non-sporulating hyphae. The brown rot fungus, *Monilia* spp. (Fungi Anamorphici), forms concentric rings of spores on fruits and *Phyllosticta* (Fungi Anamorphici) and other fungi form rings of pycnidia on leaves. The zones of spores are usually stimulated by light but may actually form during the subsequent dark periods. A strain of the fungus *Aspergillus ornatus* (Ascomycotina) reproduces asexually under light and sexually under dark and was therefore nicknamed "the bashful fungus." These examples clearly show that fungi too respond to light and dark periods.

11.1.4 Spore Liberation

A frequently observed response to light is the rhythmic discharge of spores into the environment. Spores of some fungi can be confidently identified by microscopy following impact on a sticky microscopic slide. Sampling of spores in the air of agricultural fields in England at intervals showed maximum spores of *Phytophthora infestans* (Straminipila) in the early morning, of *Ustilago nuda* (Basidiomycotina) at forenoon, of *Cladosporium* (Fungi Anamorphici) around noon and of *Sporobolomyces* (Fungi Anamorphici) during the night (Figure 11.2). In an infected apricot orchard in California, the maximum conidia of *Monilinia laxa* (Ascomycotina) occurred at night. Air sampling close to the ground of rust-infected wheat fields in Kansas showed a diurnal pattern of liberation of urediospores of *Puccinia recondita* (Basidiomycotina). The ascospores of *Sordaria fimicola* (Ascomycotina) are discharged mainly during the day and of *Daldinia concentrica* (Ascomycotina) during the night. These observations suggest that light influences maturation and discharge of spores, although the influence of temperature, humidity and wind velocity cannot be overlooked. Fungi take advantage of the conditions of light, high humidity and wind velocity to maximize the dispersal and germination of their propagules. Other examples of seasonality and photoperiodism in fungi are given by Ingold (1971) and by Roenneberg and Merrow (2003). Some examples are considered below that show the intensity (brightness of illumination), the quality (wavelength of light) and the duration (relative lengths of the alternating periods of light and darkness) that influence fungal development.

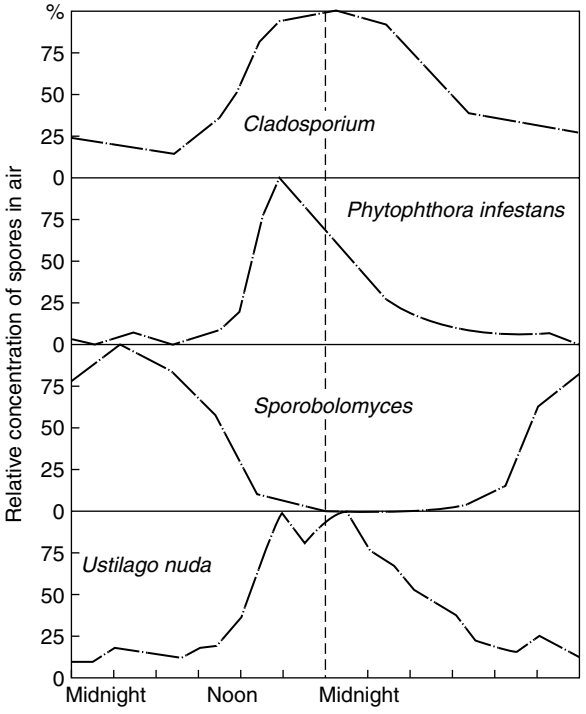

Figure 11.2 Diurnal variation in spore content in air in agricultural field in England. (From Ingold and Hudson (1993). With permission of Kluwer Academic Publishers.)

11.1.5 Phototropism

11.1.5.1 *Pilobolus* spp.

A common fungus that grows on the dung of herbivorous animals is *Pilobolus* (Zygomycotina). The dung contains nitrogen, vitamins, growth factors and minerals and satisfies the fungus' unusual nutritional requirement of a chelated form of iron. The fungus can be grown in media incorporating a decoction of dung or on a synthetic medium containing a complex iron-containing compound called coprogen. Exposure to visible light (380 to 510 nm) triggers the formation of a large bulbous cell called a *trophocyst* that is embedded in the substratum. The trophocyst elongates into a stalk about 0.5 to 1 cm high called a *sporangiophore*. The sporangiophore enlarges upwards into a crystal-clear subsporangial bulb and capped by a sporangium containing asexual spores. When dung is kept under a bell jar to provide a damp atmosphere and light, in a few days it becomes covered by turgid sporangiophores. The sporangiophore bends toward the light source and the entire black sporangium containing the spores (Figure 11.3) is shot toward light with a velocity of 5 to 10 m/s. The empty sporangiophore is thrown flat on the medium by the recoil (Page, 1962). Covering the bell jar with a black paper in which a small window has been cut demonstrates the precision with which the fungus can direct the shooting of sporangium toward a light source. The released sporangia strike the inside of the bell jar in the area that receives the illumination. This phototrophic response permits

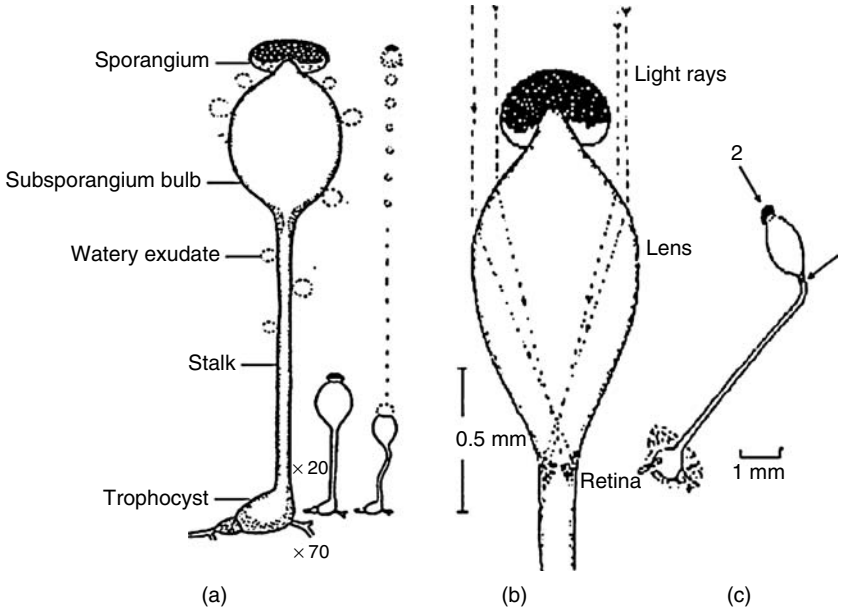

Figure 11.3 *Pilobolus kleinii*. (a) Upper part of sporangiophore acting as a simple eye. (b) Sporangiophore originally developed in light from direction 1, but two hours ago illumination was altered to direction 2. (From Ingold and Hudson (1970). With permission of Kluwer Academic Publishers.)

the sporangiophores to grow out of dung; the sporangia strike a blade of grass and adhere to it. When a grazing animal eats grass, the spores within each sporangium pass unharmed through its alimentary canal and are voided with the dung wherein they germinate.

Klein (1948) subjected *Pilobolus* grown on dung-decoction agar to light-dark (LD) cycles. He found that although sporangium formation occurred predominantly at the end of a dark period, a dark period is essential to establish periodicity of growth and maturation of fruiting bodies. Periodicities other than those observed in nature can be established by artificial illumination. Among these were light-dark cycles (in hours) not only of 12-12 but 16-16, 15-9 and 9-15. When subjected to continuous darkness, the rhythm synchronized by the previous light cycle persists and cultures initiate a self-sustained rhythm under circadian control (Uebelmesser, 1954).

The Canadian mycologist A.H. Reginald Buller suggested that the subsporangial vesicle acts as a lens, focusing the rays of light at the base of the orange-colored vesicle (Figure 11.3). The curvature results from an increase in growth in this region. A fresh crop of sporangiophores is formed daily and the discharge occurs around noon. To determine the region of the sporangiophore that is sensitive to light, Robert Page (1962) made a series of photomicrographs at intervals following unilateral illumination. The tip (about 0.5 mm) of the sporangiophore curved sharply toward a light souce in 10 minutes and reached its maximum curvature in about one hour. To respond in this way, some chemical substance must perceive light. E. Bünning (1960) compared the phototropic response of various wavelengths (action spectrum) of light by means of glass and liquid filters with the absorption spectrum of extracted pigments. From the close resemblance between the action and absorption spectrum, he suggested that the photosensitive pigment is a carotenoid. Page (1962) exposed sporangiophores to wavelengths of light dispersed by a prism and caught the shot sporangia on a glass plate (Figure 11.4). The distribution of the sporangia gave an action spectrum for phototropism. The response was strong between 410 and 420 nm. Based on the similarity of the action and the absorption spectrum, the

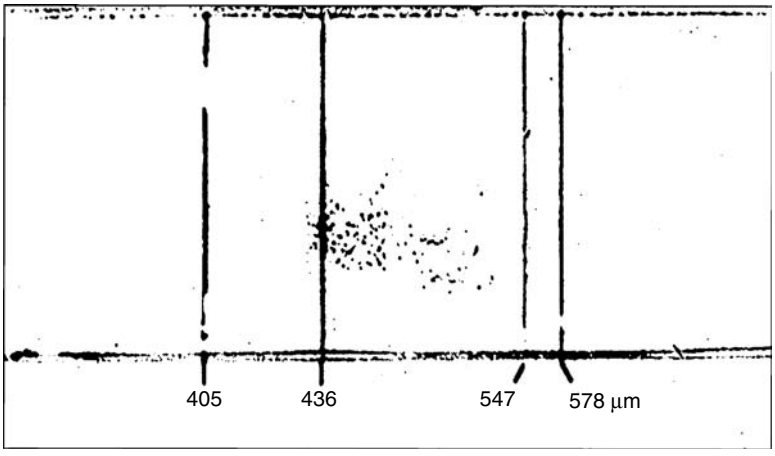

Figure 11.4 Diagram of distribution of *Pilobolus sporangia* adhering to a glass plate interposed between a culture and light dispersed by a prism. Traced from Page (1962).

photoreceptor is most likely a carotenoid or a flavin. The adaptive response of *Pilobolus* to light ensures the dissemination of its spores and the survival of the fungus.

11.1.5.2 *Phycomyces blakesleeanus*

The fungus *Phycomyces blakesleeanus* (Zygomycotina) occurs in decaying organic matter but is easily grown in laboratory media that is supplemented with thiamin. Its sporangiophore is a 10 to 15 cm long single cell (http://www.es.embnet.org/~genus/phycomyces.html), suggesting that its cell wall must be very rigid. The Nobel Laureate Max Delbrück was attracted to this fungus and left his research on phage. The sporangiophore is very sensitive to blue light (400 to 450 nm) with a threshold close to 10 photons per square micrometer, indicating that it has extremely sensitive photoreceptors. The observations suggest that the dosage of light required by the fungi is very small, allowing sporangiophores to grow out of the decaying organic matter.

When viewed from above, the sporangiophore grows counterclockwise toward the light. The blue light photoresponse indicates that the photoreceptor is a flavin or a carotenoid molecule. The bending response (Figure 11.5) is limited to the growing zone that extends from 0.1 to 2 mm below the bright yellow sporangium, darkening to nearly black (Bergman et al., 1969). From multiple exposure photographs of a sporangiophore, Dennison (1958) discovered that the sporangiophore elongates by spiral growth. To explain the phototropic oscillations of a sporangiophore about its stable equilibrium position, a three-layered wall structure was postulated. The photoreceptor is attached to a "receiving and adaptive shell" in the sporangium and twists but does not stretch. An inner wall (responsive structure) bends

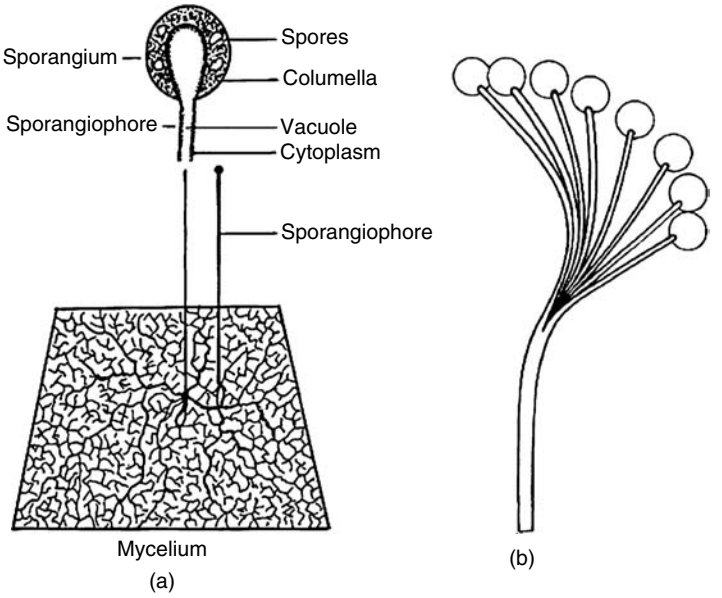

Figure 11.5 Bending of *Phycomyces* sporangiophores toward unilateral light source. Direction of illumination was from left. Tracings from photographs (Bergman et al., 1969) taken at 5 min intervals.

and grows and a "reactive structure" passively follows the bend and growth of the "inner wall" (Shropshire, 1963). These three hypothetical wall layers have not yet been related to visible structures.

A sporangiophore placed horizontally bends upwards (negative geotropism). Mutants of *Phycomyces* were isolated that are photototropically abnormal. These mutants fall into seven complementation groups, indicating that the transduction of the information from the photoreceptor to the morphogenetic response mechanism involves a number of steps. The mutants, *madA* and *madB*, are defective in gravitropic and avoidance responses. It was hypothesized that the inputs from the gravity and chemical sensors feed into the tropism path. Many physiological processes have been shown to be affected by light, including the inhibitory effect of blue light on the upward bending of a horizontally placed sporangiophore and the growth away from solid barrier (Bergman et al., 1969).

In 1976 (http://www.es.embnet.org/~genus/phycomyces.htm), Delbrück said, "I feel that if I make a serious experimental research effort (necessarily a very strenuous exercise) it should be in *Phycomyces*. I am still convinced that *Phycomyces* is the most intelligent primitive eukaryote and as such capable of giving access to the problems that will be central in the biology of the next decades. If I drop it, it will die. If I push it, it may yet catch on as phage ... caught on. Since I invested 25 years in this venture I might as well continue. I do not expect to make great discoveries, but if I continue to do the spade work my successors may do so." The difficulties in genetic analysis of *Phycomyces*, for example, the failure of stably transforming it with exogenous DNA, have dampened interest in this fungus.

11.1.6 Morphogenesis

11.1.6.1 Mushroom

Light accelerates the development of fruit bodies of the coprophilic fungus *Coprinus sterquilinus* (Basidiomycotina). Primordia appear in 8 to 11 days under continuous illumination but none appear in darkness (Madelin, 1956). Sequential light exposures are necessary to initiate and program fruit body morphogenesis in Basidiomycotina. Additional periods of illumination are required for the development of the cap, blue (400 to 520 nm) to near ultraviolet (320 to 400 nm) being most effective.

11.1.6.2 Other Fungi

Barnett and Lily (1950) reported that a strain of the fungus *Choaneophora cucurbitum* (Mucorales/Zygomycotiuna) formed conidia only in alternating light and darkness whereas another strain formed conidia in complete darkness. There are other examples of striking differences in response to light between members of the same family (Hawker, 1966). In *Penicillium isariiforme* and *P. claviforme* (Fungi Anamorphici), light triggers the development of aggregated conidiophores (coremium). In *Aspergillus nidulans*, conidiation is promoted by light of red wavelength and is reversed by far-red (Mooney and Yager, 1990). This is characteristic of the plant pigment phytochrome (a protein containing a chromophore, the light absorbing portion), which undergoes a reversible interconversion between the biologically inactive red-light-absorbing form (P_r) and the active far-red light absorbing form (P_{fr}) and controls behavior responses in plants such as seed germination, stem elongation and flowering. These fungi are attractive subjects for further experimentation.

11.2 CIRCADIAN RHYTHM IN *NEUROSPORA*

> Life is a cyclical chemical process that is regulated in four dimensions. We distinguish parts of the cycle: development describes the changes from single cell to adult, and aging the changes from adult to death. Birth to death, a cycle, and there are cycles within cycles—circannual rhythms, menstrual cycles, semilunar cycles, and daily 24 hr or circadian cycles.
>
> *Jay C. Dunlap (1999)*

> The fungi are a richly diverse collection of sessile poikilotherms. They extract what they can from their immediate environment, and are subject to the extreme conditions of nature. Thus, there is an obvious selective advantage for possessing an endogenous timing system with which to anticipate the regular, daily changes in temperature, humidity, and light.
>
> *M. Merrow, T. Roenneberg, G. Macino, and L. Franchi (2001)*

Virtually all forms of life, from unicellular bacteria to multicellular organisms including humans, exhibit the daily cycles known as *circadian* (from latin: *circa*, about; *diem*, a day). A critical feature of circadian rhythms is their self-sustained nature; that is, under constant environmental conditions they continue to repeat the daily cycle. For example, when conidia of *Neurospora crassa* (Chapter 5) are inoculated at one end on an agar growth medium in a race tube (Figure 11.6), the surface mycelium begins to grow toward the other end and after 21.6 hours the mycelium produces aerial hyphae once a day that form conidia. This cycle repeats in a regular manner every 21.6 hours. Thus, after a few days, the culture in the race tube exhibits conidiating bands alternating with undifferentiated surface growth. The time between bands (period length) is close to 24 hours

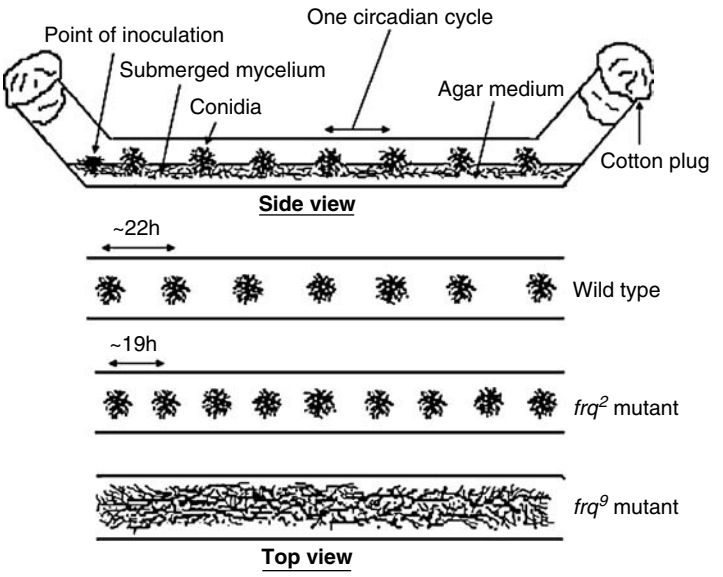

Figure **11.6** Diagram of a race tube and the *frq* mutants of *N. crassa*.

(circadian), which persists ("free runs") in the laboratory under constant illumination or darkness for several days. The rhythmic formation of conidia is a manifestation of an endogenous time-keeping system, a biological clock. This feature of *Neurospora* makes it an attractive model for investigating how living organisms keep time. In nature the rhythm is synchronized (entrained) to a 24 hour light-dark cycle. How this happens is not understood, although the light-dark cycle is undoubtedly the major factor that continuously resets the internal time-keeping mechanism.

11.2.1 A Clock Gene

Several mutants of *Neurospora* show altered period lengths of 16 to 29 hours or are *arrhythmic*, suggesting that genes affect the operation of circadian clock. For example, one mutant has a period of about 19 hours, another has a period of about 22 hours and another is arrhythmic. These mutants (Figure 11.6), frq^2, frq^7, and frq^8, respectively, are alleles of the *frequency* (*frq*) gene; their distinct phenotypes are the results of different mutations in this gene. By transformation experiments, it was found that a 7.7 kb DNA fragment from a wild strain could restore circadian rhythm in an arrhythmic mutant, allowing the cloning of a *frq* gene (McClung et al., 1989). Using the *frq* probe, Northern and Western blot analyses were made to monitor *frq* transcription and translation. The relative levels of the *frq* mRNA and FRQ protein levels cycle with a 22-hour period in the wild-type strain grown in constant darkness. *frq* is therefore the oscillator determining the conidiation rhythm. The FRQ protein has helix-turn helix DNA-binding domain and nuclear-localization motifs, which suggest that it is a transcription regulator (Bell-Pedersen, 2000).

The circadian day, about 22 hours in length, is divided into 24 equal parts: the circadian hours. A molecular model for the *Neurospora* clock (Figure 11.7) views the day in a linear fashion. By convention, CT0 corresponds to subjective dawn and CT12 to subjective dusk. The cultures maintain their rhythmicity in liquid media, making it possible to monitor changes in mRNA and protein product FRQ at different time intervals. At dawn of circadian time (CT0), both *frq* mRNA and FRQ proteins are low but *frq* transcript starts to rise at CT4. The amount of *frq* mRNA and FRQ protein cycle in circadian manner, suggesting they control a cascade of clock-controlled output genes and thereby rhythmicity of the organism. The stability of FRQ is a major determining factor for the period length of the clock. Light causes a rapid increase in the levels of *frq* transcript and resets the clock by resetting the rhythm of *frq* transcription. The FRQ protein feeds back negatively to regulate the amount of *frq* transcript. This autoregulatory loop involving the expression of genes to proteins, which in turn inhibit their own expression, is thought essential for the self-sustained circadian rhythmicity. FRQ can be considered to have a function similar to oscillator clock proteins Period (Per) and Timeless (Tim) of *Drsophila melanogaster* and CRY1 (crypto chrome) and CRY2 of mammals. The *N. crassa* clock is a generally relevant model for clock studies.

11.2.2 Regulatory Genes

In *Neurospora*, mutants called the *white collar* (*wc*) produce pigmented conidia on a collar of white mycelium and exhibit arrhythmic conidiation. They are "blind," being blocked in light-induced pigmentation (carotenogenesis) in mycelium. The finding of *frq* mRNA in a *wc-2* mutant suggested that light signaling acted primarily through *wc-1*. Cloning of *wc-1* and *wc-2* revealed that their sequences are quite similar. Both genes encode zinc-finger

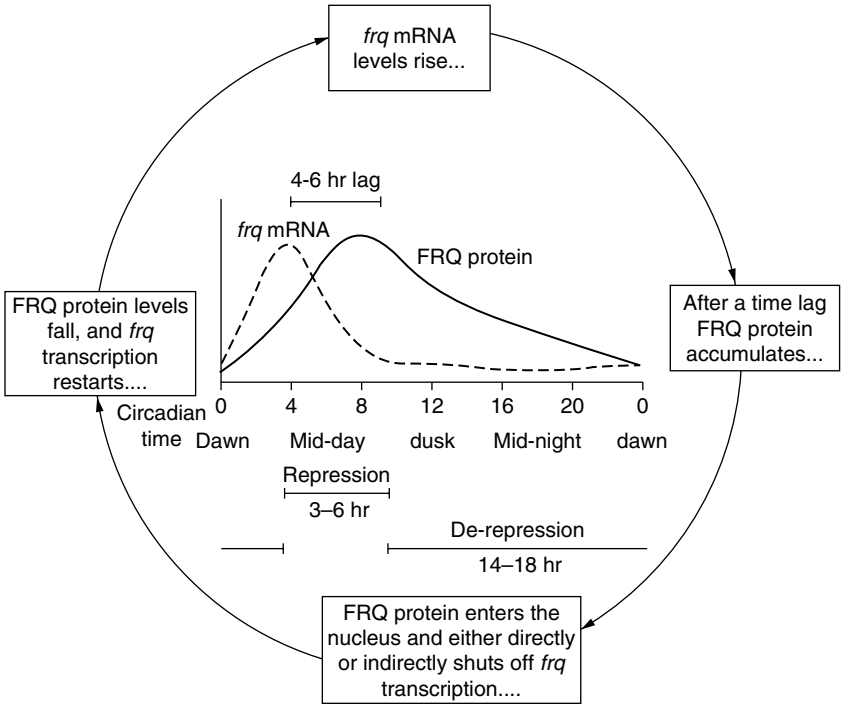
frq mRNA levels rise...
4-6 hr lag
frq mRNA
FRQ protein
After a time lag FRQ protein accumulates...
FRQ protein levels fall, and frq transcription restarts....
Circadian time
0 4 8 12 16 20 0
Dawn Mid-day dusk Mid-night dawn
Repression
3–6 hr
De-repression
14–18 hr
FRQ protein enters the nucleus and either directly or indirectly shuts off frq transcription....

Figure 11.7 Oscillations in *Neurospora* clock components. The relative amounts of *frq* mRNA and FRQ protein are plotted over 24 hours, one circadian cycle. (From Bell-Pedersen et al. (1996). With permission of Indian Academy of Science.)

transcription factors and have a "PAS" domain in common that serves as protein-protein dimerization domain found in many transcription factors and signaling components. Purified WC-1 and WC-2 form a *white collar complex*—a protein complex. (In the *Neurospora* nomenclature for proteins, the protein is given the same name as the gene but with letters in upper case without italicization.) The association of a flavin chromophore with WC-1 suggests that complex is a light-signaling molecule. Its nucleotide sequence suggests that the complex of WC-1 and WC-2 also act as a transcription factor—they bind to the frequency (*frq*) gene and induce its expression (Crosthwaite et al., 1997; Merrow et al., 2001). Null mutants of either gene are arrhythmic. A model of regulation of *frq* by white collar genes proposed by Dunlap and co-workers (Liu et al., 2000) is shown in Figure 11.8. The model envisages that FRQ plays a dual role—acting to depress its own synthesis by allowing transcription only when the concentration falls below a certain threshold and also to activate *wc-1* and *wc-2* genes that encode the DNA-binding proteins and act as transcriptional factors (positive element) in the feedback loop for the photoinduction of *frq* transcript. Phosphorylation of FRQ triggers its turnover and is a major determinant of period length in the clock. The FRQ proteins inhibit *frq* activation, making a negative autoregulatory driving feedback loop rhythm in *frq* RNA levels that is the basis of circadian rhythm in *Neurospora* and, likely, in other organisms too. The rate of degradation of FRQ is most likely the major determining factor for the period length of the clock.

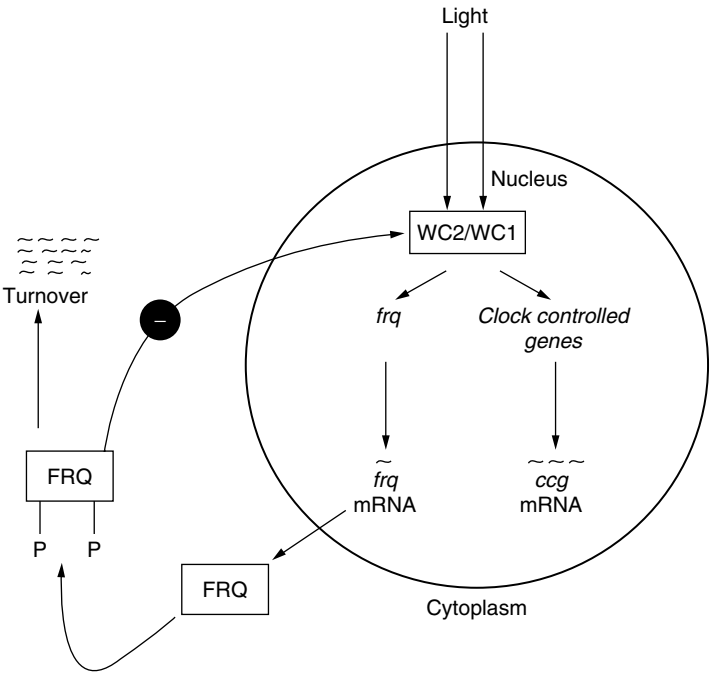

Figure 11.8 Genes and regulation of *Neurospora* clock. Adapted from Merrow and Roenneberg (2001).

11.2.3 Clock-Controlled Genes

Many genes have a role in conidiation (Chapter 7). In *Neurospora*, screening for clock-regulated genes was carried out using a subtractive hybridization of subjective morning vs. subjective evening mRNAs (Loros et al., 1989). In general, the transcripts of these genes accumulate in late night to early morning. The best-characterized clock-controlled gene is *easily wettable* (*eas*), which encodes a hydrophobic component of conidial wall and is, therefore, important for spore dissemination (Lauter et al., 1992). Conidia of *eas* are easily wettable because hydrophobin is missing. Genes associated with carotenoid biosynthesis and conidiation are also found to display a daily rhythm in mRNA accumulation (Lauter, 1996). Experiments using transcriptional profiling with DNA microarrays will provide a means to determine the full extent of clock-regulation of genes.

11.3 ENTRAINMENT

Although the endogenous rhythms are fairly constant over a broad range of temperatures, the timekeeping mechanism has the property of being reset or synchronized, known in chronobiology terminology as *entrainment*. That is, the rhythm can be advanced or delayed although the cycle has the same length, allowing the organism to coincide its clock to the day phase of environment. Entrainment is easily demonstrable in fungi: In cultures of *Pilobolus* growing in a petri dish, a single 1/2000-second high intensity light flash was

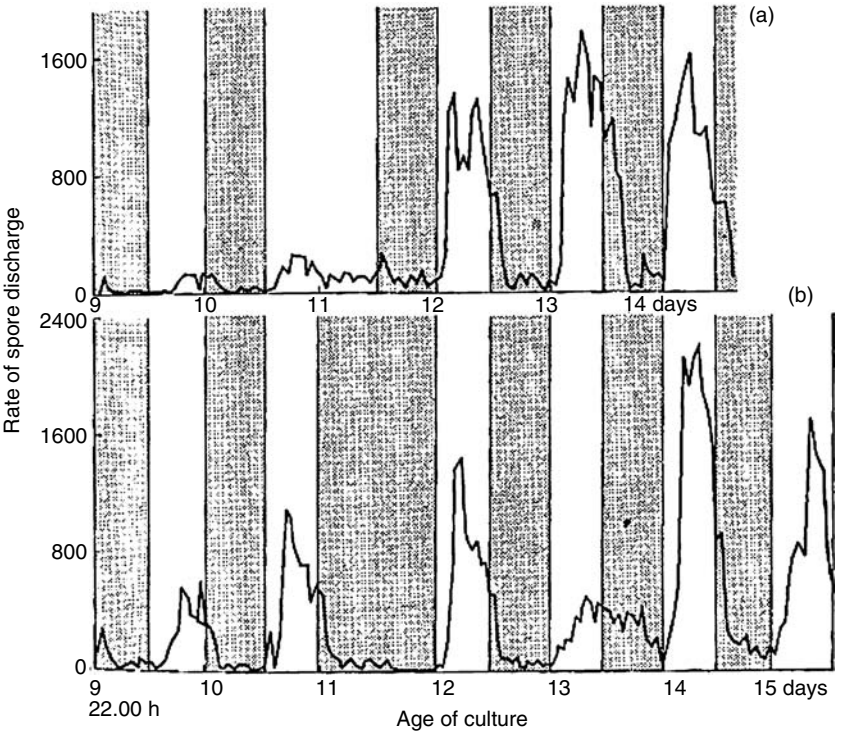

Figure 11.9 Rate of spore discharge from cultures of *Sordaria fimicola* under alternating conditions of light (unshaded area) and dark (shaded area). Cultures were grown in continuous light until day 12, and then light periods were made to correspond with "natural" period of maximum discharge for three days. The phasing of the light periods was then shifted through 12 hours by imposing either a 24 hour-period of light (a) or darkness (b). Spore discharge was immediately entrained to the new light phase. (From Austin (1968). With permission of the publisher.)

enough to rephase sporangiophore development (Bruce et al., 1960). In *Sordaria fimicola* (Ascomycotina) grown in plate cultures under alternating conditions of 12 hours of light and 12 hours of darkness, the discharge of ascospores occurred 6 hours after the beginning of the light period (Austin, 1968). The phasing of the light period was then shifted through 12 hours by imposing either a 24-hour period of light or darkness (Figure 11.9). Spore discharge was immediately entrained to the new light phase. The fact that circadian rhythms can be entrained demonstrates they are adaptive. The biochemical mechanism of adjustments remains a mystery.

11.4 SUMMARY

In the natural habitat, it is difficult to distinguish between the effects of many variables such as temperature, light, aeration and humidity on growth and reproduction but sufficient experiments with pure cultures under controlled conditions have demonstrated that light has a strong influence, chiefly in the timing of the production and discharge of spores—the

most efficient means of dispersal and migration of species. As the given examples become widely known, more investigators will be prompted to study the effect of light in fungal development and more such fungi are bound to be discovered in the future. The significance of these responses is not obvious but, fungi being largely terrestrial organisms, these events must in some way be linked to daily cycles of light and darkness for optimal fitness and survival. A surprising number of homologues to blue and red light-sensing genes have been discovered in a terrestrial fungus such as *Neurospora* (Borkovich et al., 2004). At present, little is known of the mechanism of the perception of light into reproduction. In *N. crassa*, rhythmic response can be quantitated, it is amenable for genetic analysis and it is extremely well-suited for the analysis of complex phenomenon of biological rhythm. As the Earth rotates and revolves round the sun, the daily cycle of light and dark in different regions varies annually with latitude. The studies of conidiation rhythm in *Neurospora* collected from different latitudes could provide clues into the role of circadian sporulation rhythm. Which part of the mycelium perceives light, and what is the molecular identity of the photoreceptor and its cellular localization, are among other questions awaiting answers. A breakthrough has come from the genetic approach that has identified certain genes underlying the timekeeping mechanism. The product of the frequency locus contributes to a molecular oscillator whose rate of degradation is a major determining factor for the period length of the circadian clock. At present, the model of circadian rhythm in this fungus envisages transcription of *frq* gene(s), followed by production of FRQ protein(s), their feedback on self-transcription, degradation of FRQ protein(s) releasing the negative feedback, allowing a new round of transcription and resulting in molecular oscillations of RNA and protein. Among important research goals is the identification of genes regulated by *frq* and the signaling pathways from the environment through which the cellular clock is synchronized to the external world. The "race tube" system of easily visualizing a rhythm in conidiation in *Neurospora crassa* and screening genetic mutants affected in periodicities makes *Neurospora* one of the most attractive models for approaching studies of rhythmic activities in plants and animals showing higher complexity of cellular organization.

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Chapter 12

Decomposition of Biomass

From the crib to the funeral pyre, man relies on plant cell walls as the basis for his shelter, clothing, fuel and fodder for his animals, boats, baskets, his musical instruments and for the paper on which he writes poetry or scribbles his philosophy.

The material commonly referred to as *biomass* is mainly the plant cell wall that forms the bulk of the organic matter produced from photosynthetic fixation of carbon dioxide by green plants. Biomass is comprised of about 35% cellulose, 25% hemicelluloses and 35% lignin, approximating to 100 gigatonnes of lignocellulose-rich plant cell wall material being produced annually. Fungi, through their ability to penetrate inside the dead tissue via grooves and pits in the cell wall, are responsible for returning to soil a large quantity of nutrients for plant growth that would be unavailable otherwise. The fungi are thus effective scavengers playing a significant role in recycling nutrients and in biogeochemical processes in the biosphere. Their co-evolution with plants, animals and other biota is only beginning to be appreciated.

12.1 DECAY OF WOOD AND LITTER

Since fungal hyphae have a large surface to volume ratio, fungi are particularly effective agents in decomposing the biomass—though strictly speaking, communities other than fungi comprised of bacteria and actinomycetes also participate in this process. Cellulose and hemicelluloses are the chief polysaccharides in plant cell walls which in woody tissues are encrusted with lignin. Most fungi living as saprophytes on organic matter produce cellulases and hemicellulases. However, the occurrence of a fungus where cellulose or lignin is abundant is not enough to infer that the fungus is responsible for decomposing these polymers, since a non-cellulolytic fungus may live commensally on products formed by a cellulolytic fungus. The removal of certain plant cell wall polysaccharides by one species may improve the accessibility of another species to cellulose (polysaccharide composed of glucose molecules joined in β-1,4 linkage) or hemicelluloses (non-cellulose polysaccharide, composed of β-linked pentose with side chains). Direct observation of sporulating structures and cultural (plating) techniques demonstrate that the decomposition of biomass involves activities of a mixed microflora comprising fungi, actinomycetes and bacteria.

Studies with pure cultures of fungi show that the enzymes involved in biomass degradation are synthesized in significant amounts only when inducing substrates are

present. How the insoluble substrates induce the synthesis of the enzymes requires more study. Fungi belonging to the Basidiomycotina are the most important organisms contributing to the decay of wood. Their leathery or woody fruit bodies (basidiocarps) project out from tree trunks (Figure 12.1), signaling that their mycelium inside the plant tissue has been slowly attacking lignin and polysaccharide constituents. The spores or mycelia of these fungi gain entry into wood through wounds. They are carried by wind, water, insects, birds or by rodents that feed on and breed inside the weakened or dying trees. The early stages of decay of standing or fallen trees is due to several species but soon competition between mycelia sets in and a single individual mycelium can extend to several meters (Boddy and Rayner, 1982). Decay of biomass is an aerobic process requiring oxygen for the oxidation of lignin phenols. Moisture is vital not only for the synthesis of

Figure 12.1 Wood decay. Fruit bodies (basidiocarps) protruding from a decaying fallen tree in a rainforest. Wood-rot fungi often attack the standing tree. (Photo: Jean Paul Ferrero/Ardea.) (See color insert following page 140.)

Table 12.1 Chemical Analysis of Decayed and Sound Wood Samples[1]

Fungus	Cellulose (% dry wt)	Lignin (% dry wt)
Coniophora puteana	36.1	58.8
Phanerochaete chrysosporium	79.1	69.9
Trametes versicolor	76.3	43.1
Trametes versicolor	85.3	29.6
Control birch wood	84.2	43.3

[1]http://www.geocities.com/Research Triangle/Lab/2819/WHITE.htm

fungal protoplasm but also for the swelling of the substrate and the diffusion of the digestive enzymes into the substratum through pits and pores.

The decay of timber is monitored by a chemical extraction procedure. Lignin is quantitatively extracted by chlorite-acetic acid. Hemicelluloses are extracted as alkali-soluble material, whereas cellulose is insoluble in alkali. Extractions and estimations of these materials show that the various wood-decay fungi differ in their abilities to digest lignin, cellulose and xylan (Table 12.1). The hyphae grow in intimate contact with the cell wall, enzymatically fragmenting it and transforming the decayed plant material into humus with a high phenol content. Fungi cannot use lignin as the sole carbon source; the presence of a metabolizable carbohydrate, from which hydrogen peroxide can be generated, is essential for the growth of fungus and for depolymerization of lignin.

12.1.1 White-Rot and Brown-Rot Fungi

The presence in wood of lignin—a complex phenylpropanoid polymer that is the most recalcitrant of organic compounds—renders wood decay a very slow process. The fungi referred to as the white-rot fungi produce polyphenol oxidase and laccase that oxidize phenol compounds, giving the wood a bleached or pale appearance and transforming it into a fibrous mass. Electron microscopy of decaying wood using $KMnO_4$ as a fixative and stain shows the greatest electron density in the middle lamella. Once lignin is removed, the middle lamella between cells is degraded and the cells separate (see Blanchette, 1991). In contrast, the brown-rot fungi by some undiscovered mechanism circumvent the lignin barrier and utilize hemicelluloses and celluloses, leaving lignin essentially undigested. The wood breaks into pieces that crumble into a brown powder. A method of distinguishing between the brown and the white rot fungi is the Bavendamm test, based on the formation of a brown color in the medium when phenolics are oxidized. The fungi are grown on an agar medium containing phenolic compounds. The white rot fungi produce polyphenol oxidase that shows a dark zone around mycelium. The brown rot fungi do not produce polyphenol oxidases and do not show any coloration.

12.1.2 Litter Decomposers

The microorganisms bringing about decomposition of litter are identified by plating decaying tissue macerates on suitable nutrient agar or by the direct observation method

of the tissue incubated in damp atmosphere and identifying these on the basis of morphology. Among the soil-inhabiting fungi—*Penicillium* (Fungi Anamorphici), *Humicola* (Fungi Anamorphici), *Trichoderma* (Fungi Anamorphici), *Mucor* (Straminipila), *Collybia* (Basidiomycotina), *Hydnum* (Basidiomycotina), *Marasmius* (Basidiomycotina), *Mycena* (Basidiomycotina) and others—are typical litter-decomposing fungi. Scanning electron microscopy of tissue surfaces allows the identification of fungi that are actually growing on the plant litter. The colonization appears to be largely superficial. The rate of decomposition of leaf litter is markedly dependent upon the rate at which litter fragments, a result of the activity of small soil animals such as earthworms, slugs, millipedes and mites that ingest plant litter (Dix and Webster, 1995). Fragmentation increases the surface area for microbial attack, though little is known of the role of individual species. In temperate regions the agarics are among the most active agents of decomposition. They are active producers of laccases or polyphenol oxidases which detoxify litter phenolics. However, litter decomposition in tropical forests has been little studied.

12.2 BIOCHEMISTRY OF DEGRADATION OF CELL WALL POLYMERS

12.2.1 Lignin Degradation

Lignin is formed by random cross-linking of three monomer phenylpropanoid units: *p*-hydroxycinnamyl (coumaryl) alcohol, 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol and 3,5-dimethoxy-4-hydroxycinnamyl (sinapyl) alcohol with several different carbon-carbon and carbon-oxygen linkages (Figure 12.2). Lignin encrusts the cellulose microfibrils within plant cell walls, giving the vascular plant the rigidity and protecting the plant from weather, insects and pathogenic organisms. It is remarkable, therefore, that a few fungi belonging to Basidiomycotina, such as *Trametes versicolor*, *Bjerkandera adusta*, *Hypholoma fasciculare*, *Stereum hirsutum*, *Gymnopilus penetrans*, *Agaricus bisporus*, *Pleurotus ostratus* and *Lentimus edodes* (http://www.ftns.wau.nl/imb/research/wrf.htnl), can selectively degrade lignin and access the carbohydrate polymers within the cell wall.

The screening of active ligninolytic fungi is done by inoculating wood blocks with the fungus and estimating lignin loss by chemical analyses and transmission electron microscopy. Lignin-degrading activity is measured by measuring the evolution of $^{14}CO_2$ from ^{14}C-labeled synthetic lignin prepared by polymerizing ^{14}C-labelled *p*-hydroxycinnamyl alcohols with horseradish peroxidase, or by the oxidation of a lignin model compound, veratryl (3,4-dimethoxybenzyl) alcohol to veratraldehyde in the presence of H_2O_2. Electron microscopy of wood decayed by white-rot fungi revealed that lignin is degraded at some distance from the hyphae, suggesting that the hyphae produce a highly reactive oxygen species that diffuses out and depolymerizes lignin by breaking the carbon-oxygen and the carbon-carbon bonds. The inability of large-size enzymes to diffuse into wood suggests that fungi employ smaller reactive oxygen species that cleaves C–C bonds. An extracellularly produced lignin-degrading enzyme resembling peroxidase (haem protein) in spectral properties was isolated from *Phanerochaete chrysosporium* (Basidiomycotina). Spectacular photographs of this fungus may be viewed online (http://botit.botany.wisc.edu/tomes_fungi/may97.html). The fungus grows optimally at about 40°C and has been isolated from stored wood chips used for the manufacture of paper and in saw-mill waste that attains high temperatures. The extracellular enzyme

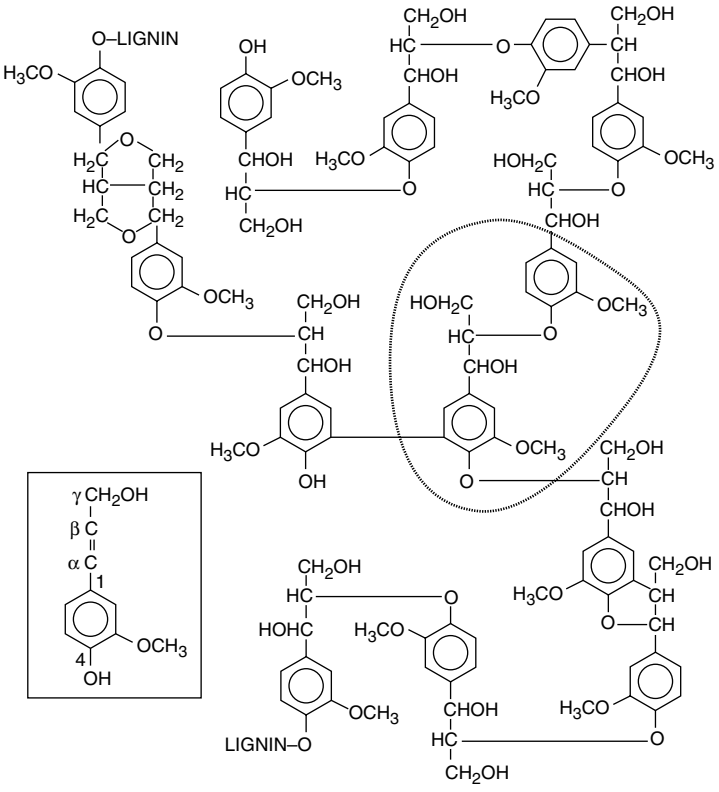

Figure 12.2 Structure of lignin. Inset shows coniferyl alcohol, the phenylpropanoid building block. The major arylglycerol-ß-aryl ether structure is circled (From Hammel (1997). With permission of CAB International.)

catalyzed C–C cleavage in lignin model compounds (Tien and Kirk, 1984) and required hydrogen peroxide for activity. Peroxidases catalyze reactions wherein hydrogen peroxide is reduced, while a substrate is oxidized simultaneously,

$$AH_2 + H_2O_2 \rightarrow A + 2H_2O$$

where AH_2 is a reduced substrate and A is the oxidized substrate. Hydrogen peroxide is a powerful oxidant and may be produced by oxidases that oxidize sugars to sugar lactones (Hammel, 1997). Immunochemical localization using gold-labeled antiserum in sections of white-rotted wood shows that lignin peroxidase is present in cell walls undergoing delignification. Two types of ligninase have been found: those which require manganese for catalytic activity (MnP) and those that do not require a metal ion for activity (LiP). The enzymes can oxidize aromatic compounds containing free phenolic groups by removal of one electron from the aryl rings to form an aromatic cation radical. (A *radical* is a molecular fragment having one or more unpaired electrons. It pairs up with other electrons to make new chemical bonds, making radicals highly reactive). The radicals break down the lignin polymer and explain how lignin is degraded at sites some distance away from the hyphae. In white rot fungi, the extracellular H_2O_2 required for the activity of lignin peroxidases is produced from the oxidation of glyoxal and methylglyoxal—metabolites

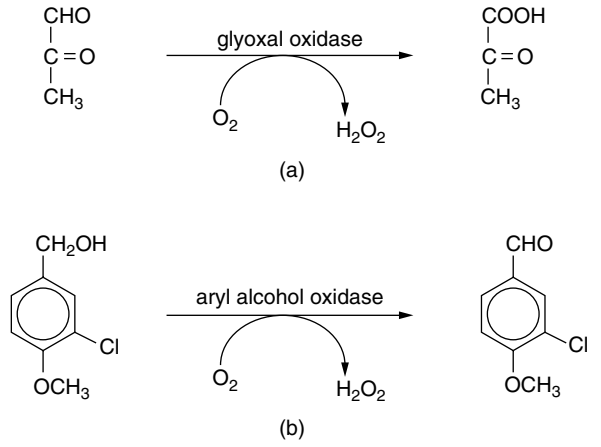

Figure 12.3 Production of extracellular H_2O_2 by wood rotting fungi. (From Hammel (1987). With permission of CAB International.)

secreted by the fungus (Figure 12.3). Wood decay by white rot fungi requires the cooperative action of lignin peroxidases and glyoxal oxidase. White-rot fungi synthesize and secrete veratryl alcohol, a substrate for its own peroxidase enzymes.

In liquid-grown *P. chrysosporium* cultures, ligninolytic activity appeared after primary growth of the fungus ceased due to nutrient limitation (Jeffries et al., 1981), raising the question whether lignin degradation is a growth-associated process. A different picture emerged using a technique that allowed for simultaneous monitoring of growth as well as secretion of enzymes by the hyphae. Moukha et al. (1993) grew the fungus on an agar medium sandwiched between two perforated membranes (Figure 1.9). This arrangement allowed the free uptake of nutrients, autoradiographic detection of growth by the application of radioactively labeled acetylglucosamine (a chitin precursor) and the detection of secreted enzyme using lignin peroxidase antibodies. Though the radial growth of the fungal colony stopped, new short branches were initiated at the colony center that secreted Mn^{2+}-dependent lignin peroxidase. The results suggested that although primary growth of the fungus was over, nonetheless, secondary branches were produced, apparently by the recycling of intracellular metabolites. In surface-grown cultures, this period coincided with accumulation of RNA transcripts and secretion of ligninase. Electron microscopy, cytochemistry and immunogold labeling of *P. chrysosporium* growing in wood show that lignin degrading activity (LiP and MnP) is associated with the mucilage (glucan) sheath at the apical regions of the hyphal cell-wall surface (Daniel et al., 1989; Daniel et al., 1990; Ruel and Joseleau, 1991).

12.2.2 Cellulose Degradation

Cellulose is the most abundant organic compound in nature. It is a homopolymer of 10,000 or more D-glucose units in β(1-4) glycosidic linkage (Figure 12.4). This linkage results in the aggregation of the flat glucan chains, side-by side and above each other. Hydrogen bonds between the ring oxygen atom and the hydroxyl groups of glucose molecules result in the tight aggregation of the glucan chains in microfibrils. Their packing is so ordered

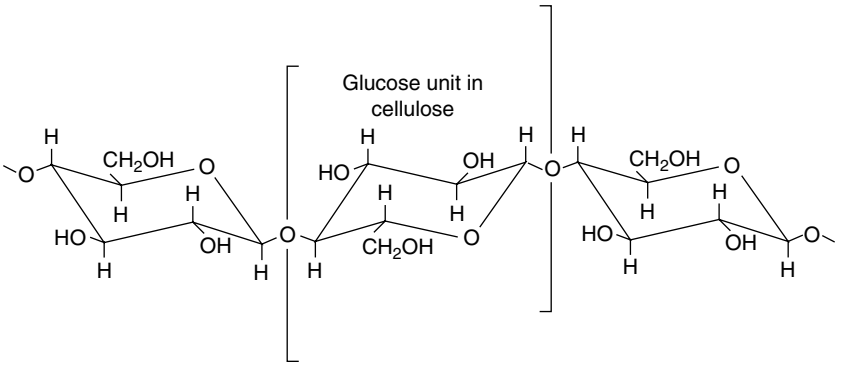

Figure 12.4 Cellulose, a polymer of β1→4 linked glucose.

that cellulose exists primarily in the crystalline state (Figure 12.5) and is impermeable to water and it is, therefore, generally resistant to microbial attack.

Since cellulose is insoluble, the utilization of cellulose by fungi is viewed as a problem of converting the insoluble material into soluble sugars for transport within the mycelium and one that could be translated for converting cellulose into glucose. Scientists at the US Army Natick Laboratory in Natick, Massachusetts, envisaged a practical process for the production of glucose from cellulosic material (wood waste and wastepaper) and its fermentation into ethanol for use as biofuel (Mandels, 1975). In the 1970s, when the energy crunch became acute, a world-wide program started for screening and selecting fungi that secreted mixtures of exo- and endocellulase and β-glucosidase in culture medium. Strains of *Trichoderma* were developed through mutagenesis and selection that, under optimized conditions, secreted up to around 30 grams of cellulase enzyme per liter of the

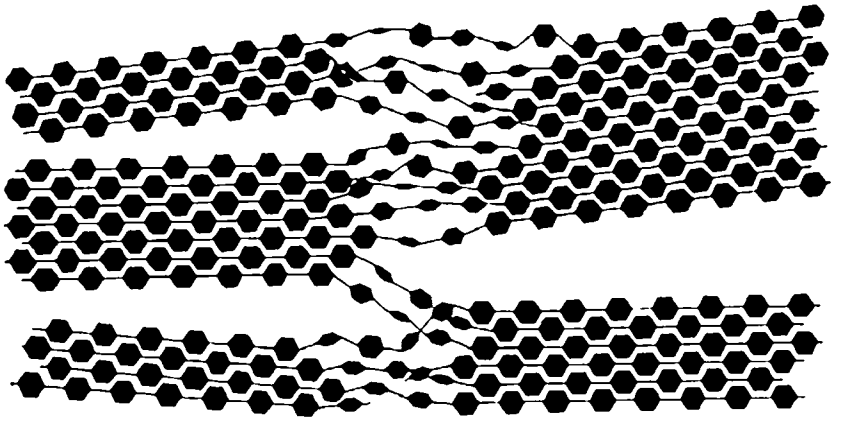

Figure 12.5 Diagram of cellulose showing crystalline and amorphous parts due to tight or loose packing of glucose chains. (From Meyer, B.S. and Anderson, J.B. (1950), *An Introduction to Plant Physiology*, Van Nostrand.)

culture medium, generating much euphoria over the possibility of developing an industrial process for converting cellulosic material into glucose and ethanol.

A few species of fungi were found that degraded cellulose completely under the growth conditions (Bhat and Maheshwari, 1987). Commonly, three types of hydrolytic enzymes are found in culture filtrates of cellulolytic fungi: (i) endoglucanase, or 1,4-β-D-glucan glucanohydrolase (molecular weight 25 to 50 kDa), which cleaves β-linkages at random, commonly in the amorphous part of cellulose; (ii) the exoglucanase or exo(1,4)β-glucanase (molecular weight 40 to 60 kDa), which releases cellobiose from the crystalline parts of cellulose; and (iii) the β-glucosidase (molecular weight 165 to 182 kDa), which releases cellobiose and short chain cellooligosaccharides. One of the most active fungi capable of utilizing cellulose rapidly and completely is a thermophilic fungus *Sporotrichum thermophile* (Figure 12.6). Its rate of cellulose utilization in shake-flask cultures is even faster than of *Trichoderma viride*, even though its secreted levels of endoglucanase and exoglucanase enzymes are lower. This raised the question whether endo- and exocellulases and β-glucosidase are the primary enzymes required for extracellular solubilization and utilization cellulose. In experiment, the growth of *Sporotrichum thermophile* in shake-flask cultures was stopped by the addition of cycloheximide, a protein synthesis inhibitor. Even though the endoglucanase, exoglucanase and β-glucosidase secreted prior to the inhibitor treatment were in the growth medium, the utilization (solubilization) of cellulose in the culture medium was interrupted. It was hypothesized that some crucial factor(s), replenished by growing of the mycelium, are involved in the utilization of cellulose, implying that cellulose degradation is a growth-associated process. The cellulose-grown culture filtrates of *Sporotrichum thermophile* had limited action under the *in vitro* conditions. Light microscopy showed that action of culture filtrate enzymes on Whatman filter paper (a substrate made from cotton, commonly used in experiments with cellulose enzymes) was initiated at naturally existing "disjointed regions" in the cell wall of

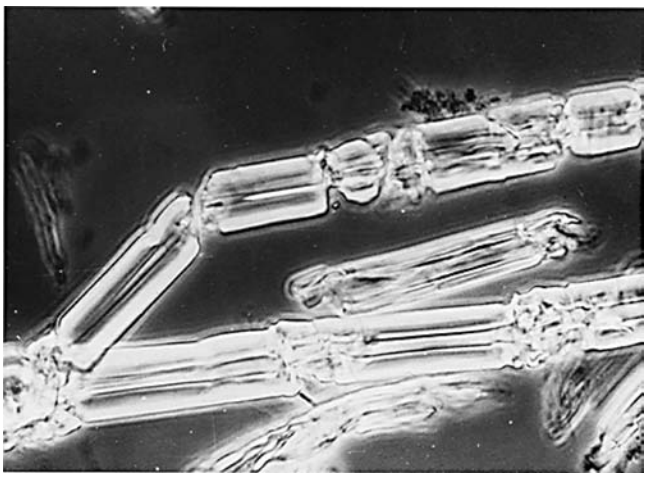

Figure 12.6 Photomicrograph of cellulose (filter paper) fiber treated *in vitro* with culture filtrate of a cellulolytic fungus *Sporotrichum thermophile*. Courtesy of M.K. Bhat.

elongated cells. The concentrated cell-free culture filtrates shortened the cellulose fibers and caused the splaying of the cell wall (Figure 12.7), although solubilization (i.e., production of reducing sugars or glucose) was limited.

A reason for the limited solubilization of cellulose under *in vitro* conditions is end-product inhibition of cellulase activity by glucose and cellobiose that, under the growth conditions, are constantly removed by the organism. The cell-free culture filtrates of some fungi, such as *Trichoderma viride* (Fungi Anamorphici), *Humicola insolens* (Fungi Anamorphici), *Chaetomium thermophile* (Ascomycotina) and *Thermoascus aurantiacus* (Ascomycotina), solubilize cellulose much more rapidly under aerobic conditions than under anaerobic conditions, indicating that an oxidative reaction is involved in breakdown of cellulose. An oxidative enzyme, cellobiose dehydrogenase, is present in some fungi, which in the presence of Fe^{2+} can generate reactive hydroxyl radicals, $H_2O_2 + Fe^{2+} \rightarrow HO\bullet + Fe^{3+}$. These radicals, in cooperation with cellulase, can depolymerize cellulose (Mansfield et al., 1997). $H_2O_2 + Fe^{2+}$, known as the Fenton's reagent, is thought to loosen cell-wall structure and allow the diffusion of enzymes (Koenigs, 1974a,b; Kirk and Farrell, 1987). Although a fundamental process in nature, our knowledge of the mechanisms involved in cellulose decomposition is far from complete.

In culture conditions, approximately 50% of the carbon in the growth medium is used in the production of new biomass. Yet in nature, a vast quantity of litter is decomposed by fungi year after year without visible accumulation of mycelia. A distinctive feature of

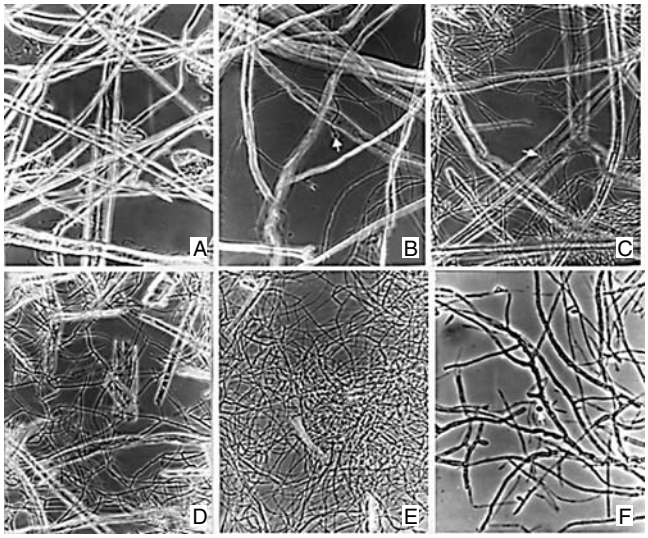

Figure 12.7 Utilization of cellulose by *Sporotrichum thermophile*. Light microscopy of samples inform shake-flask cultures of *Sporotrichum thermophile*. (A) Initial appearance of fibers. (B) 16-hour culture showing germination of conidia and no perceptible changes in structure of cellulose fibers. (C) 30-hour culture showing extensive fragmentation of fibers. (D) Magnified view of fibers from 30-hour samples showing fragmentation at "weak spots" (↑). (E) 48-hour culture showing extensive fungal growth and nearly complete utilization of cellulose. (F) 72-hour culture showing total utilization of cellulose fibers and beginning of sporulation (↑). Courtesy of M.K. Bhat.

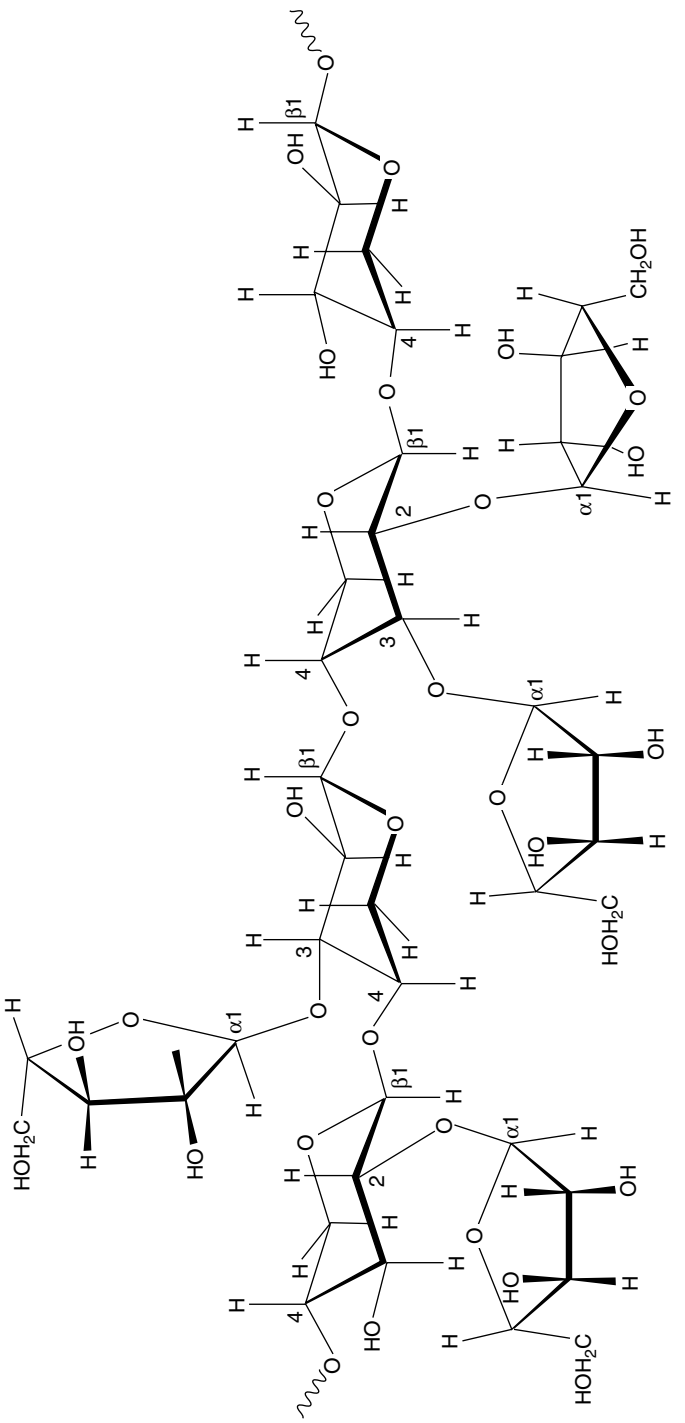

Figure 12.8 Xylan. A polysaccharide composed of five carbon xylose sugar. C-1 of one five-carbon sugar ring is linked to C-4 of another sugar ring by a β-glycosidic bond.

the development of litter fungi on cellulosic substrates is the precocious differentiation of hypha into spores and the autolysis of hyphal cells. This may explain how in nature vast quantities of cellulose is constantly recycled but without the accumulation of fungal mycelium. Cellulose has a strong effect on fungal reproduction and spores are incorporated into the soil—a significant content of "soil" is fungal spores. Polymeric substrate and its depolymerized forms affect the morphology of the fungus differently (Gaikwad and Maheshwari, 1994).

12.2.3 Hemicellulose Degradation

Hemicelluloses (non-cellulosic polysaccharides) are composed of β,1-4 linked pentose with side chains consisting of sugars, sugar acids and acetyl esters that prevent the aggregation of chains as in cellulose. Hemicelluloses are hydrogen-bonded to cellulose and covalently-bonded to lignin. Commonly occurring hemicelluloses are pectin, xylan, arabinan and rhamnogalactouranan. Next to cellulose, xylan (Figure 12.8) is the most abundant structural polysaccharide in wood cell walls. The complete hydrolysis of xylan requires cooperative action of the endoxylanases that randomly cleave the β-1,4-linked xylan backbone, the β-xylosidases that hydrolyze xylooligomers and the different branch-splitting enzymes that remove the sugars attached to the backbone, e.g., glucuronic acid and arabinose. Xylanases of fungi show a multiplicity of forms with molecular mass ranging from 20 to 76 kDa. Analysis of the breakdown products of delignified cell walls using xylanase sequentially and simultaneously with cellulase show that a mixture of these enzymes is more effective in degrading cell wall than individual enzymes (Prabhu and Maheshwari, 1999).

12.3 CONCLUDING REMARKS

The process of decomposition of biomass is highly complex, involving the activities of a mixed microflora and fauna. One of the difficulties in understanding of the mechanisms in decomposition of biomass is that we do not know the structure of the plant cell wall—the chief constituent of the biomass—which varies from plant to plant and changes with age and from season to season. An "old" hypothesis that wood-decay fungi employ extracellular reactive oxygen species and oxidoreductase enzymes to cleave lignocellulose is receiving renewed attention. Clues may come from the study of the morphology and physiology of a fungus when growing and degrading an insoluble polymer than when it is growing on their derived (soluble) products. To date, studies have concentrated on the degradation of polymers in shake-flask cultures and the role of adhesion of microorganisms to substrate has received little attention. The mucilage sheath associated with fungal hyphae in material decaying *in vivo* may be important because considerable enzyme activity could be located at the surface of hyphae. Few studies have compared the rates of biomass decomposition by pure cultures with those with mixed cultures. Finally, it is quite seriously suggested to engineer a single organism or to use a mixed culture of organisms with the ability to utilize cellulose and hemicelluloses, and for utilization of the resulting products for conversion into ethanol to use as a fuel. This otherwise attractive scheme could have disastrous consequences if it leads to deforestation due to the harvesting of trees and plants for cellulose fibers.

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Part VI

Populations

Chapter 13

Species, Their Diversity and Populations

> Knowledge of a flute or a kettledrum is not sufficient to understand all the other instruments in a symphony orchestra or to predict their characteristic. Nor is knowledge of a single species, however complete, adequate for understanding diverse species. Diversity of research in the laboratory must at least dimly reflect the diversity of species in nature if the scope and beauty of evolutionary improvisations are to be appreciated and the genetic manipulations that brought them about are to be understood.
>
> *David D. Perkins*

Fungi live in very diverse habitats and display great diversity in structure and biochemistry. Table 13.1 gives a sample of terms used to denote their diversity. The number of scientists with expertise in isolating and identifying fungi is rapidly dwindling. Our knowledge of fungi is therefore heavily biased on forms that are recognized unambiguously in nature, or of fungi that can be isolated from nature and induced to sporulate in pure culture. We know much less about the distribution of species in space and in time. Although the era of "model organisms" and of genomics is drawing attention away from the study of fungal populations, this may be a temporary phase because of the urge "to go places" to sample fungi for analyzing populations. Sooner or later, a student of fungal biology is confronted with the following questions. (i) What is meant by species? How can the number of species of fungi in nature be estimated? (ii) Of what value is fungal diversity? (iii) How can the similarities and variation between individuals within a population and the limits of such a population be determined? (iv) What are the possible causes of population changes and, ultimately, of evolutionary changes? How can the relative contributions of sexual and asexual modes of reproduction be ascertained in those fungi that possess both modes of reproduction? (v) How can the homology (affiliations) of confusing forms be resolved?

13.1 VALUE OF DIVERSITY

13.1.1 Applied Research

Most marketable biotechnology products still result from the traditional approach of isolation and screening of microbes from natural habitats and the exploitation of natural variability in populations. All biotechnology begins with a preferred fungal genome drawn from nature—a natural species and its numerous genetic strains. Some examples of inter- and intraspecies differences in enzyme productivity and properties of enzymes of commercial value can be

Table 13.1 Some Terms That Depict the Special Habitat and Diversity of Fungi

Term	Meaning	Common example(s)
Anthropophilic fungi	Infectious only to man	*Trichophyton rubrum*
Aquatic fungi (water molds)	Fungi resident in aquatic habitats	*Saprolegnia*
Brown rot fungi	Fungi which rot wood giving it a dark brown color	*Fistulina*, *Daedalea*, *Coniophora*
Coprophilous fungi	Fungi growing on dung of herbivore animals	*Pilobolus*, *Podospora*, *Coprinus*
Corticolous fungi	Fungi growing on tree bark	
Dermatophyte	Fungi that live as parasites on skin, hair, or nails of man and other animals	*Trichophyton interdigitale*, *Microsporum canis*, *Arthobotrys* sp.
Edible fungi	Fungi used as food	*Agaricus campestris*, *Pleurotus versicolor*
Endophytic fungi	Symptomless parasitic fungi in mutualistic association with living plants	*Balansia* sp., *Curvularia* sp.
Entomogenous fungi	Insect-parasitizing fungi	*Entomophthora*, *Cordyceps*, *Septobasidium*, *Beauveria*
Hypogeous fungi	Fungi growing below ground	*Tuber* sp.
Keratinophilic fungi	Fungi growing on feathers, horns	*Onygena equine*, *Nannizia*
Lichen forming fungi	Fungal symbiont of lichen thallus	*Cladonia cristellata*, *Xanthoria* sp., *Phaeographina fulgurata*
Marine (saprobic) fungi	Fungi growing and sporulating in marine or estuarine habitats	*Dendryphiella salina*, *Mycosphaerella*
Mesophilic fungi	Fungi thriving between 10–40°C	Vast majority of fungi, e.g., *Aspergillus niger*
Mildew	Fungi producing whitish growth on living plants	Downy mildew (*Peronospora viticola*), powdery mildew (*Erysiphe graminis*)
Mycorrhizal fungi	Fungi in symbiotic association with living roots	Mostly basidiomycetous fungi belonging to the families Agaricaceae, Boletaceae
Mycoparasites	Fungi parasitic on other living fungi	*Trichoderma* spp., *Piptocephalius* sp., *Gliocladium roseum*
Necrotrophic fungi	Parasitic fungi that kill host cells in advance of its hyphae and derive their organic nutrients from the dead cells	*Pythium*, *Monilinia fruticola*, *Penicillium expansum*
Nematophagous (Predacious) fungi	Fungi parasitic on nematodes	*Arthobotrys* sp., *Dactylaria* sp.
Osmotolerant fungi	Fungi capable of growth in solutions of high osmotic pressure	*Aspergillus restrictus*, *A. flavus*, *A. amstelodami*
Phylloplane fungi	Fungi growing on aerial surface of living leaves	*Cladosporium herbarum*, *Alternaria alternata*

Table 13.1 (*Continued*)

Term	Meaning	Common example(s)
Psychrophilic fungi	Fungi growing at <10°C, maximum temperature of growth at 15–20°C	*Mucor* sp., *Fusarium nivale*, *Typhula idahoensis*
Pyroxyrophilous fungi	Fungi growing on burnt trees, wood or burnt areas of the ground	*Anthracobia* sp., *Pyronema* sp., *Daldinia* sp.
Rumen fungus	Anaerobic chytridaceous fungus	*Neocallimastix frontalis*
Rust fungi	Obligate biotrophs causing reddish brown pustules on plants	*Puccinia graminis*, *Uromyces* spp.
Sewage fungi	Fungi growing in polluted waters	*Leptomitus lacteus*, *Fusarium aqueductuum*
Soft rot fungi	Fungi which cause a watery rot of parenchymatous tissue	*Penicillium expansum*, *Monilinia fruticola*
Sooty molds	Fungi which cover the leaves as black sooty mass	*Meliola* sp.
Sugar fungi	Fungi which utilize only simple organic compounds, lacking the ability to decompose complex organic materials.	*Aspergillus* sp., *Penicllium* sp., *Mucor* sp.
Thermophilic fungi	Fungi which can grow at 45°C or above but not at 20°C	*Thermomyces lanuginosus*, *Mucor miehei*
Water molds	Fungi found in waters	*Saprolegnia*, *Achlya*, *Dictyuchus*
Wilt fungi	Fungi causing wilt of plants, trees	*Ceratocystis fagacearum*
Xerotolerant fungi	Fungi growing on jams, salty foods at <0.85 aw	*Aspergillus fumigatus*, *Cladosporium* sp.

found in Maheshwari et al. (2000). Designing culture media for the selective enrichment and isolation of desired types and manipulating environmental conditions (temperature, aeration, pH, osmotic pressure) from an infinite number of permutations and combinations of environmental variables is a challenge.

13.1.2 Basic Research

> Because of biological diversity, no single species can serve as a universal model. Each species has its peculiar strengths and limitations.
>
> *David D. Perkins (1991)*

The enormous diversity of fungi provides enough opportunities to biologists to choose fungi suitable to investigate a diversity of problems. It is through biochemical dissection of the wealth of genetic diversity that we will appreciate the fundamental design principles of living organisms. The currently favored fungi in research are given in Table 13.2.

Table 13.2 Currently Favored Fungi in Research

Fungus	Research area
Ascobolus immerses	Meiotic recombination
Ashbya gossypii	Nuclear migration and positioning
Aspergillus nidulans	Conidial differentiation, nuclear migration, cytokinesis
Aspergillus flavus	Mycotoxins
Aspergillus niger	Enzymology and metabolism
Candida albicans	Pathogenecity, dimorphism
Colletotrichum graminicola	Topographical signals in pathogenesis
Coprinus cinereus	Morphogenesis of multihyphal structures
Fusarium oxysporum	Transposons
Magnaporthe grisea	Mechanisms in pathogenecity
Neurospora crassa, *N. intermedia*, *N. tetrasperma*	Hyphal growth, chromosomal rearrangements, function of *het* genes, mating type genes, meiotic drive, epigenetic phenomena, biological rhythm, mitochondrial biogenesis, vesicle trafficking, developmental genetics, biochemical regulation, speciation
Phanerochaete chrysosporium	Biochemistry of lignin degradation
Phycomyces blakesleeanus	Photobiology
Podospora anserina	Senescence, mitochondrial genetics, prions
Saccharomyces cerevisiae, *Schizosaccharomyces pombe*	Cell cycle regulation, cell polarity, protein secretion, biotechnology
Saprolegnia ferax	Hyphal morphology and growth
Ustilago maydis	Mating recognition, cell signaling, pathogenesis
Uromyces phaseoli	Surface signaling in pathogenesis

13.2 NUMBER OF FUNGAL SPECIES

Previously, a species was defined as a group of individuals having common morphological characters. Based on the degree of discrimination adopted by the taxonomist—a scientist who identifies and classifies according to a nomenclature and classification system—approximately 70,000 species of fungi have been described based on morphological features such as the structure of conidiophores, the color and the method of formation of spores, the types of ascocarps, the features of basidium and many others. However, this number is considered to be grossly lower than the total number of fungal species. Hawksworth (1991) estimated the number of species of fungi based on the ratio between the species of vascular plants and the species of fungi in well-studied regions of the world. For the United States, this ratio is 1:1; for Finland 1:4; for Switzerland 1:4; and for India it is 1:0.5. The best-studied region is the British Isles, where the ratio is 1:6. These ratios show us the regions that require mycological studies. For example, the ratio for India is undoubtedly low because of the underexploration of the subcontinent's alpine, aquatic, arid and tropical environments. If the ratio of one fungus to six plants is applied to the global total of 250,000 species of vascular plants, the total number of fungal species comes close to 1.5 million. Only 5% of this estimated number of fungal species are actually documented. Where are the undiscovered fungi to be found? Unusual fungi lurk in unusual ecological niches and habitats (Subramanian, 1992).

Devising methods for their enumeration is difficult. Traditional isolation and enumeration techniques use the soil dilution plates, which in essence involve adding soil or a diluted suspension to petri dishes and covering with a suitable agar medium. It is estimated that there are up to a million fungal spores in a gram of dry soil. Some species can have unusual nutritional requirements and therefore uncultivable on media commonly employed and unidentifiable. There is no simple solution for estimating species diversity.

13.3 SPECIES RECOGNITION

13.3.1 MSR, BSR and PSR

The vast majority of the 70,000 described fungi were identified based on morphological characters. However, fungi are notorious for their phenotypic plasticity—a characteristic that led Buxton (1960) to comment that fungi are "a mutable and treacherous tribe." Individuals may show striking nongenetic variation depending on changes in the growth environment. Thus, forms that may in fact be closely related could be given the status of different species. On the other hand, a group of individuals could share certain morphological characters but, in fact, be genetically isolated. Therefore, for species recognition, morphological criteria (*morphological species recognition*, or MSR) alone are not sufficient. A species has been defined variously (Taylor et al., 2000) as "groups of actually or potentially interbreeding populations which are reproductively isolated from other such groups" or "the smallest aggregation of populations with a common lineage that share unique, diagnosable phenotypic characters" or "a single lineage of ancestor-descendant populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate." An excellent example of species discriminated by mating tests (*biological species recognition*, or BSR) is provided by the fungus *Neurospora*. Shear and Dodge (1927) showed that by mating tests, the morphological species *Monilia sitophila* can be split into three species: *N. crassa*, *N. sitophila* and *N. tetrasperma*. However, the identification of a species mating test is not easy to apply as in approximately 20% of all fungi, grouped as Fungi Anamorphici, the sexual stage either does not occur or the conditions under which it occurs are not known. The use of a mating test (reproductive success in crosses) in species identification is therefore of limited use.

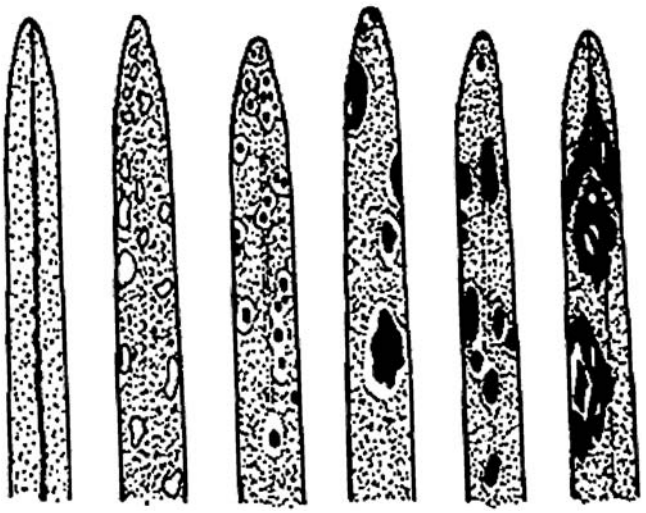
With the recombinant DNA technology, long stretches of DNA can be cloned and sequenced and compared to determine whether two nucleic acid molecules are similar. Nucleic acid sequences are scanned and fitted by computer models into a branching tree pattern based on maximum parsimony. A software program is used to measure the summed average index of resemblance between fungi and arrange them into a branching tree diagram, i.e., a tree that unites the specimens having the most features in common to demonstrate the relative relationships in a group of individuals, with adjacent branches depicting the greatest or closest genetic similarity (Burnett, 2003). A phylogenetic system that classifies organisms according to their evolutionary sequence, that is, enables one to determine at a glance the ancestors and derivatives, is used in recognizing species. It is particularly useful in those organisms where the mating test cannot be applied. In this phylogenetic diagram, the branch points or nodes reflect divergence from a previously common sequence and the length of a branch or distance is a measure of the mean number of estimated character changes (substitutions) required to convert one sequence to another. A group of organisms is identified that is an *out-group*—an independent

evolutionary lineage, qualifying as a reproductively isolated species (*phylogenetic species recognition*, or PSR). In *Neurospora*, correspondence existed between groups of individuals identified as species by BSR and PSR criteria; however, PSR provided the greater resolution (Dettman et al., 2003). Data from six loci (*al-1*, *frq*, *gpd*, *mat a*, *mat A* and ITS/5.8S rRNA) consistently suggested that among the five outbreeding species recognized on the bases of MSR or BSR, *N. discreta* diverged first. More species have been found by the criterion of PSR than by MSR or BSR.

13.4 DISCOVERY OF INTRASPECIES VARIABILITY

13.4.1 Physiological Races

Before the molecular techniques, the concept of variation was provided by E.C. Stakman and his associates who noted that a variety of crop plant bred for resistance to a particular species of a rust fungus failed to remain resistant to that particular fungus. They demonstrated that the species of the black (or stem) rust fungus, an obligate parasite on wheat and grasses (namely *Puccinia graminis* f.sp. *tritici*) could be subdivided into over 200 "physiological races." These varied in virulence characters; namely, the size and shape of lesions produced on leaves of different varieties of wheat called the "tester" varieties (Figure 13.1), although they were indistinguishable in morphological features such as the size of urediospores. The recognition of physiological races explained why no variety of wheat plant bred for resistance to a pathogen is permanent because a new physiological race can arise through sexual recombination. *Puccinia graminis* f.sp. *tritici* is heteroecious,



Author:Two labels for "0", please verify. Is 0; correct? Would 0a be better?

Figure 13.1 Infection types produced by wheat stem rust, *Puccinia graminis* var. *tritici*, on differential varieties of wheat. 0, Entirely immune. 0;, Practically immune. 1, Extremely resistant. 2, Moderately resistant. 3, Moderately susceptible. 4, Completely susceptible. Tracing from Stakman and Harrar (1957).

that is, it has a parts of its life cycle on wheat and the other on an alternate host, barberry. When this was recognized, a barberry destruction program was undertaken in the United States to control rust epidemics with some limited success as new races of wheat rust continue to arise through mutation in the absence of sexual reproduction.

The application of pathogenecity tests show that intraspecific groups of individuals restricted to a characteristic host (*forma speciales*) is a common feature of several plant pathogenic fungi. For example, *forma speciales* have been discovered in *Phytophthora infestans*, in the powdery mildew (*Erysiphe* spp.), in the downy mildew (*Peronospora* spp.) and in *Fusarium oxysporum*.

13.4.2 Vegetative Compatibility

Since the majority of species are non-pathogenic, the pathogenecity test for detecting intraspecies variation used for the rust fungi is of limited application. In the pre-molecular era, the formation of heterokaryon, involving hyphal fusion between strains followed by mixing of their protoplasm, was a common method to determine their genetic relatedness. When two test strains are paired opposite to each other on agar media, frequent anastomoses occur between the hyphae of two closely related fungal strains. If not, the mycelia of the test strains confronted on nutrient media show an antagonistic reaction consisting of vacuolated, dying and often pigmented hyphal cells between them called the *barrage reaction*. Fusion of hyphae is controlled by genes called *heterokaryon incompatibility* (*het*) loci or *vegetative incompatibility* (*vic*) loci. For example, 11 and 8 *het* loci in *N. crassa* and *Aspergillus nidulans*, respectively, and 10 *vic* loci in *Fusarium oxysporum* have been identified. The hyphae of two fungal strains fuse to form a homogeneous mycelium (heterokaryon) only if the alleles at each of the corresponding *het* or *vic* loci are identical. This method is more reliable if the test strains have genetic markers whose defect can be remedied by complementation. In one of the procedures, the test strains are grown in media containing chlorate. The spontaneous nitrate non-utilizing (nitrate reductase) mutants as chlorate-resistant mutants are selected. These strains are paired against each other on minimal media plates with nitrate as the nitrogen source. Strains of the same vegetative compatibility group (VCG) form prototrophic heterokaryons whereas the strains of different VCG are incapable of forming prototrophic heterokaryons. Based on this test, Jacobson and Gordon (1990) resolved a morphologically identical population of the muskmelon wilt fungus *Fusarium oxysporum* f.sp. *melonis* into several incompatible groups and demonstrated intraspecies variability in this facultative parasite. This method has also been used to detect variation in the population of honey fungus, *Armillaria mellea* (Basidiomycotina), in Australia. The vegetative compatibility test reflects closeness but not the genotypic identity of the individuals.

13.5 GENERATION OF VARIATION

13.5.1 Mutation and Heterokaryosis

Ultimately all variations arise from mutations. Although a rare event, of the order of 10^{-6} per nucleus, mutation assumes greater importance in fungi than in other organisms because the coenocytic fungal mycelium contains thousands or perhaps millions of nuclei. The effect of mutation on phenotype may not be detectable because of masking of the mutant nuclei by the wild-type nuclei in the heterokaryon. It is only when the mutant nuclei segregate as

uninucleate spores and germinate to produce a growth variant containing descendants of the mutant nuclei that the mutation is revealed (Chapter 2). Such a variant may differ morphologically from the original strain (Adhvaryu and Maheshwari, 2000). Chapter 3 refers to the finding that a single multinucleate spore of mycorrhizal fungi contains genetically different nuclei. More than one ITS sequence has been obtained from a single multinucleate spore. Since sexual reproduction is unknown in these fungi, heterokaryosis is believed to play an important role in the variability of Glomales (Sanders, 1999).

13.5.2 Transposable Elements

Transposable elements are insertion sequences in DNA that have an intrinsic capability of transposing within the host genome. They contribute to genetic variation by both modification of gene sequence and modification of chromosome structure through translocation, deletion and duplication and have been identified in fungi (Daboussi, 1996). Transposons are present in multiple copies and consequently can be discovered through DNA sequencing. The plant pathogenic fungi *Fusarium oxysporum* and *Mycosphaerella graminicola* are typical examples where various types of transposons have been discovered.

13.6 DETECTION OF GENETIC VARIATION IN POPULATIONS

Burnett (2003) described a *population* as a pattern of distribution of individuals with distinctive but comparable morphology and genotypes. A basic requirement for the study of populations is that the fungus is recognized in nature and enough samples are collected over a wide range of geographical distances for inferences. A fungus suited admirably for population studies is *Neurospora* (Ascomycotina) since it produces distinctive orange conidia that allow its practically unambiguous recognition in nature. Practical techniques have been devised for sampling, transportation and reliable identification of its species in the laboratory (Perkins and Turner, 1988; 2001). Over 4000 samples have been collected globally and collections preserved, providing the most valuable resource material for population studies (Turner et al., 2001). For fungi whose mycelium is in soil, e.g., *Fusarium oxysporum* (Fungi Anamorphici), the fungus is isolated from soil by dilution plating on a selective medium (Gordon et al., 1992). For fungi immersed in substrate such as wood, blocks can be taken and plated to isolate the fungus. Using this method, Rayner (1991) and colleagues detected different basidiomycete individuals, demarcated by interactive zones of lines in a single branch of an oak tree.

13.6.1 Isozymes

Variation in strains can be due to variants of proteins that catalyze the same reaction but differ in net charge due to differences in their amino acid composition and can therefore be resolved by electrophoresis. The protein extracts of samples are subjected to polyacrylamide gel electrophoresis and the difference in mobilities of a specific enzyme are compared after visualization following a specific staining reaction. Such variants, called *isozymes*, imply diversity in the amino acid sequences of the proteins analyzed and of the genes that encode them. Spieth (1975) surveyed *N. intermedia* for general proteins (acid phosphatases and esterases) and detected a high degree of genetic variability among natural populations. This technique is widely used to detect variation in all forms of life. The technique ultimately detects variation among DNA sequences that code for isozymes.

The late blight fungus *Phytophthora infestans* (Straminipila) collected world-wide was long considered a single asexual clone. However, populations from the highlands of central Mexico were more variable than collected elsewhere and the isozyme analysis was used to demonstrate genetic diversity in populations. The finding of protein polymorphism in *P. infestans* isolates was consistent with the discovery of heterothallism and sexual reproduction, suggesting that this region is the site of origin of this fungus In the 1970s and 1980s, the analysis of isozymes of glucose-phosphate isomerase in populations of the *P. infestans* from Europe, the United States and Canada suggested an intercontinental spread of the fungus from Mexico to Europe through the transport of diseased potato tubers. As a result of man-mediated migration of strains on diseased crops, sexual reproduction elsewhere has become potentially possible, alerting the plant pathologists to protect potato crops by a continuous program of resistance breeding.

Section 13.4.1 referred to the control of wheat rust in the United States by the eradication of barberry, the alternative host of this heterothallic fungus on which sexual reproduction occurs (see Appendix). Protein markers and pathogenecity tests were used to investigate the role of sexual recombination in maintaining variability within the population of *Puccinia graminis* f.sp. *tritici*. By 1975, there was a notable difference in the number of physiological races as the sexual stage had ceased to function. In regions where barberry is absent, like Australia, the pathogen population has more limited isozyme and virulence phenotypes than in regions where opportunities for sexual reproduction exist (Burdon and Roelfs, 1985).

13.6.2 RFLP

Restriction fragment length polymorphisms (RFLP) are based on the hybridization of DNA probes to fragments of DNA produced by cutting with specific restriction endonucleases and size fractionated by electrophoresis. RFLPs (Figure 13.2) are used to detect variation in nucleotide sequence among homologous sections of chromosomes due to restriction-enzyme recognition-site changes along the section of chromosome where the probe hybridizes. Because RFLPs can detect variation in both coding and noncoding regions of DNA, they are much more variable than isozymes and therefore more useful markers (Figure 13.3). RFLP and isozyme methods have been commonly used to analyze populations and to recognize subdivisions within these populations (Table 13.3). The RFLP technique is a convenient method to study long-range dispersal patterns of plant pathogenic fungi and suggested the airborne dispersal of the wheat stem rust fungus from India and Africa to Australia across the Indian Ocean.

13.6.3 RAPD

In a modified technique called *random amplified polymorphic DNA* (RAPD), synthetic 10-base long primers are used to amplify random sequences of genomic DNA from the test strains by the polymerase chain reaction. Variation is detected as the presence or absence of amplified DNA sequences. RAPD polymorphisms were detected in 10 random isolates of *Erysiphe graminis* f.sp. *Hordei*, which causes the powdery mildew of barley, sampled from a single field. The DNA fingerprints (Figure 13.4) were different, suggesting that these isolates were clonal lineages. In a study of the ectomycorrhizal fungus *Suillus granulatus*, Jacobson et al. (1993) demonstrated the high resolution of RAPD marker

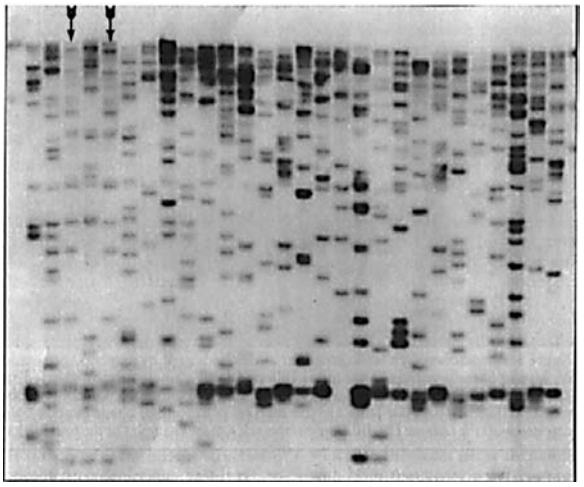


Figure 13.2 RFLPs of 28 *Mycosphaerella graminicola* isolates from a single infected wheat field. The DNA extracted from isolates was separated by gel electrophoresis and hybridized to a cloned radiolabelled DNA fragment (probe) segment and X-ray film was exposed to the gel. Arrows indicate isolates having the same DNA fingerprints are products of asexual reproduction. (From McDonald and McDermott (1993). With permission of the publisher.)

analysis. Some of the isolates of the fungus, earlier reported as similar based on vegetative compatibility reaction and considered to belong to same genotype, were found to be genetically dissimilar by RAPD analysis. This method resolved 17 collected strains of *Aspergillus niger* into 15 subgroups (Megnegneau et al., 1993). The RAPD method has a finer resolution than either RFLP or isozymes.

Figure 13.3 Autoradiodiagram of hybridization of a DNA probe with size fractionated DNA fragments of same 28 *Mycosphaerella graminicola* isolates as in Figure 13.2. The first and last lanes are markers. The numbers designate examples of four alleles. (From McDonald and McDermott (1993). With permission of the publisher.)

Table 13.3 Variation in Fungal Populations

Species	Data type	Geographic scale	No. of populations	No. of isolates
Agaricus bisporus	RFLP	Intercontinental	2	342
Mycosphaerella graminicola	RFLP	Local	3	512
Neurospora intermedia	Allozyme	Intercontinental	4	145
Schizophyllum commune	Allozyme	Intercontinental	7	136

13.6.4 Ribosomal DNA

The nuclear ribosomal coding cistron (rDNA) has been widely utilized for detecting variation among isolates of a fungus. The rDNA is composed of tandemly repeated units, each unit being composed of 5S, 25S and 18S rDNA (Figure 13.5). Two noncoding regions exist in each repeat, the internal transcribed spacer (ITS1 and ITS2) and an intergenic non-transcribed spacer (NTS). The NTS can be preferentially amplified by PCR using primers, cut with restriction enzymes and the RFLPs separated by electrophoresis and compared.

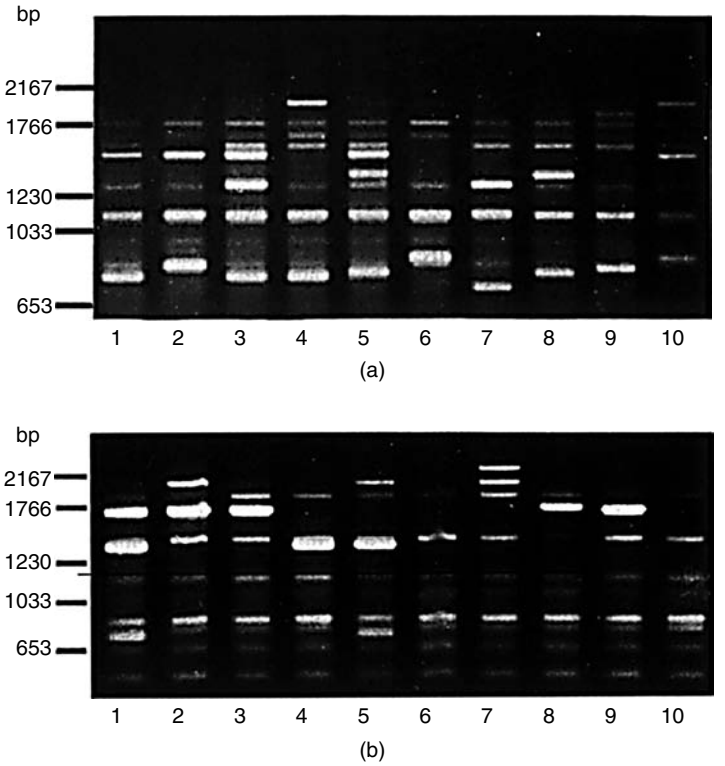


Figure 13.4 RAPDs in ten random isolates of *Erysiphe graminis* f.sp. *hordei* sampled from a single population using two different 10-base primers (above and below). (From McDonald and McDermott (1993). With permission of the publisher.)

Figure 13.5 Basic organization of one unit of ribosomal DNA.

The fruiting bodies of the split-gill mushroom fungus *Schizophyllum commune* are readily recognized on natural woody substrates and this tissue collected for isolation and analyses of genomic DNA (James et al., 2001). Using the primer sequences annealing to the 5S and 18S rDNA genes, the intragenic spacer regions were amplified by PCR technique and analyzed for molecular variation. Of the 195 strains collected from different geographical regions, 145 haplotypes (distinct DNA fingerprints) showed unique IGS1. The sequence data showed that populations from the Eastern hemisphere, North America, South America and the Caribbean are genetically discrete.

13.6.5 Mitochondrial DNA and Mitochondrial Plasmids

Not all variations are due to changes in nuclear DNA. Within a fungal species, mitochondrial DNA shows a high level of variation at the population level, detected as restriction length polymorphisms (RFLPs). In addition, natural populations of fungi have a variety of linear and circular plasmids in mitochondria in mitochondria (Table 13.4). Some of these plasmids integrate into mitochondrial DNA and cause the death of strains that harbor these plasmids (Griffiths, 1995). By simple subculture tests, natural populations of *Neurospora* have been broadly classified into senescence-prone and immortal strains (Chapter 14).

13.6.6 DNA Sequence

The DNA sequence approach basically involves comparing nucleotide sequences of specific segments of DNA. The sequences are aligned to determine the numbers of differences between comparable molecules. This allows the measurement of similarities and differences between individuals, to determine how they may have evolved and in how much time their genomes diverged from another from a common ancestor. Nucleic acid sequence data is the ultimate level for determining homology. Computer programs fit the data

Table 13.4 Mitochondrial Plasmids in Naturally Occurring Strains of *Neurospora intermedia*[1]

Plasmid	Structure	Size (bp)
pVARKUD	Circular	3675
pVARKUD SATELLITE	Circular	881
pFIJI	Circular	5268
pLABELLE	Circular	4070
pHARBIN	Circular	4.9 kb
pKALILO	Linear	8642

[1]http://pages.slu.edu/faculty/jennellj/plasmids_nc.html

graphically in the form of branching trees on the principle of parsimony, i.e., closely related individuals have more similar sequences of a particular molecule and are, therefore, more closely related. The branch points (forks) represent the most recent ancestor common to all species beyond that point.

13.6.7 Karyotype Polymorphism

Although the small size of the fungal chromosomes (between 0.2 and 10 Mb of DNA) has been a deterrent in cytological studies, their small sizes can be resolved by pulsed field gel electrophoresis (Chapter 4) and therefore provide another potentially useful marker for analysis of variation. Chromosome length polymorphism—in the form of variation in chromosome size or number—is detected among field isolates of pea root pathogen, *Nectria haematococca*, from diverse geographic origin. Variability was marked among minichromosomes, smaller than 2 Mb. Five isolates of the wheat-blotch pathogen *Mycosphaerella graminicola* had noticeably different karyotypes (McDonald and Martinez, see: Kistler and Miao, 1992).

13.6.8 Spore Killer Elements

Spore killer elements in Ascomycotina are genes that cause the death of ascospores that do not contain the killer (Sk^k) elements. In *Neurospora*, distribution of Spore killer allele in population can be made simply by crossing tester strains that are sensitive to the killer (Turner, 2001). The strains to be tested are used as a male parent and each group of octads (eight ascospores) in the sides of the cross tubes are examined under a dissecting microscope as to whether they are comprised of all black or four black and four white (aborted) ascospores. Spore killer elements are known in three other fungi: *Podospora*, *Gibberella* and *Cochliobolus* (Raju, 1994). In natural populations of *N. intermedia*, non-killer strains are frequent; killer strains are found only in samples collected from Borneo, Java and Papua New Guinea.

13.7 SPECIATION

Speciation is the process of origin of a new species. For example, *N. intermedia* occur on burned substrates in tropical and subtropical areas in the Eastern and Western Hemispheres. A type found almost exclusively on nonburned substrates (for example, ontjom, a food item made by inoculating pressed soya or peanut cakes with *Neurospora*, or on corn cobs) in the Eastern Hemisphere is yellow rather than pinkish-orange. The yellow "ecotype" is distinct also in its conidia (size and nuclear number), habitat and ecology. Although the two types can be coerced to mate in laboratory conditions, yet there is no evidence that because of the geographical isolation the two types are members of an interbreeding population. The phylogenetic trees constructed based on variation in the nontranscribed spacer suggested that the yellow isolates are a separate lineage, distinct from a larger *N. crassa*/*intermedia* clade (Figure 13.6). No definite phylogeny was apparent other than *N. discreta* was divergent from all other species. Rather, the yellow type is on the threshold of evolving into a distinct species. Though gene homology is identified more precisely than homology based on morphological characters, nevertheless caution is necessary in drawing inferences from molecular studies or phylogenetic trees constructed based on single locus.

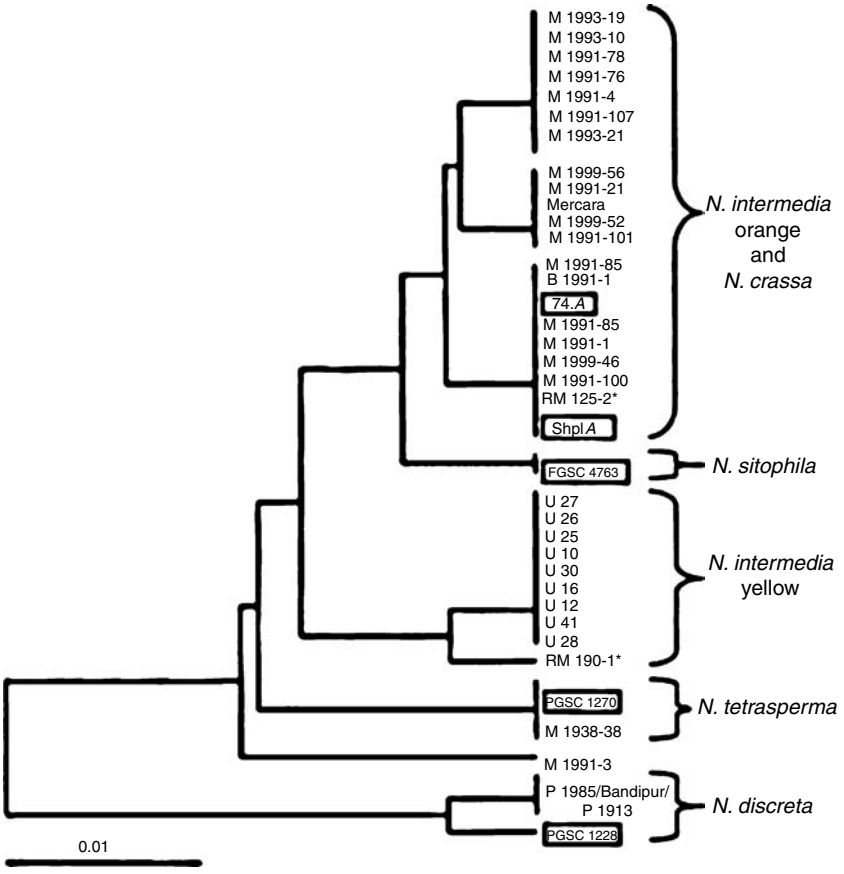


Figure 13.6 A phylogenetic tree of five heterothallic species of *Neurospora* collected from India. The phylogram is based on variations in the non-transcribed spacer of DNA. Standard species testers based on BSR are boxed. Asterisks are local species testers. The yellow ecotype of *N. intermedia* formed a monophyletic group. The scale indicates genetic distance. (From Adhvaryu and Maheshwari (2002).)

13.8 CONCLUDING REMARKS

Although some see the twenty-first century as the age of model organisms, some see every species as a source of new knowledge and advocate study of fungi representing different phylogenetic groups and different ecological situations to understand the diverse ways different organisms have solved the problems of existence (Perkins, 1991). Though deceptively simple with mycelium hidden in substratum, at the molecular level the degree of variability in fungi is similar to that displayed by highly complex organisms such as man, mice or flies. Although the numbers of asexually produced spores far exceeds those produced by sexual reproduction, the fungal populations are predominantly sexual. The study of intraspecies diversity provides insights into the role of production of sexual and asexual spores, the mode of spread and survival of fungal pathogens, mechanisms in

generation and maintenance of variation and the adaptive mechanisms in fungi. It has led to recognition of mitochondrial plasmids, transposons and the discovery of senescing strains. Molecular markers allow the determination of phylogenetic relationships among individuals within and between subpopulations and charting the course of the evolution of species, the understanding of biochemical design and adaptation to particular habitats. With the advent of recombinant DNA methodology, a new era in biotechnology has begun but new products and processes still result from the traditional approach of isolation and exploitation of variants in populations.

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Chapter 14

Senescence

> The fungi … are progressive, ever changing and evolving rapidly in their own way, so that they are capable of becoming adapted to every condition of life. We may rest assured that as green plants and animals disappear one by one from the face of the globe, some of the fungi will always be present to dispose of the last remains.
>
> *B.O. Dodge*

A type of growth in mushroom fungi (Basidiomycotina) occasionally seen in pastures or grasslands is known as the "fairy ring" (Figure 14.1). It refers to a circular development of aerial fruiting bodies (basidiocarps). According to a myth, the circular pattern of basidiocarps represents the path of fairies dancing in the night. Actually, the fairy ring develops from the perennial, subterranean mycelium that extends outwards and produces ephemeral fruit bodies year after year in the shape of a ring. From the rate of expansion of the diameter of these fairy rings, the age of the mycelium in some cases is estimated to be several hundred years old. In Chapter 1, we learned about a colony of *Armillaria bulbosa* that is more than a millennium old. Fungi are therefore regarded as potentially immortal organisms. Their immortal nature is manifested by their continuous propagation by transfer of a small amount of vegetative mycelium to agar medium. Infrequently, however, some fungi deteriorate and die upon subculturing. These strains provide valuable material for investigating mechanisms in aging and death.

14.1 DISCOVERY OF SENESCING STRAINS

Since *Podospora* and *Neurospora* (Ascomycotina) are commonly used in genetical research, their stocks are maintained by regular subculturing. Not surprisingly, the phenomenon of *senescence* is best documented in these two fungi. In the mycelium of *Podospora anserina*, the mycelium grows to a limited extent following the germination of ascospores. Therefore, senescence in this species is a part of its normal development. On the other hand, *Neurospora* is potentially immortal. Some stocks of this fungus used by Beadle and Tatum over fifty years ago (Chapter 5) are still alive after numerous subcultures. However, nearly 30% of strains of *N. intermedia* collected from the island of Kauai in Hawaii and from Maddur in peninsular India have died in 5 to 50 subcultures. Initially, a senescent strain is morphologically indistinguishable from a long-living strain. However, as subculturing continues, the quantity of aerial mycelium is reduced, conidia are not formed, the respiratory activity diminishes and levels of cytochromes decrease

Figure 14.1 A fairy ring in grassland. Photo courtesy Angela B. Shiflet.

(Figure 14.2). Eventually, growth ceases completely and the culture is regarded to have died. Thus, if a new isolate is likely to be used for investigation of senescence phenomenon, parallel subcultures from the same strain should be metabolically immobilized by lyophilization, storage in anhydrous silica gel or by cryopreservation.

14.2 DISTINGUISHING NUCLEUS AND MITOCHONDRIA BASED SENESCENCE

14.2.1 Genetic Cross

Since DNA is present not only in the nucleus but also in mitochondria, whether the factor that determines senescence is in the nucleus or in the mitochondria can be determined from its mode of inheritance. When two parents are crossed, generally the cytoplasm (mitochondria) from the maternal (female) parent is transmitted to the progeny; rarely is there transfer of cytoplasm from the paternal parent (male). For example, in *P. anserina*, the cross (exceptional) non-senescent female × senescent male yielded non-senescent progeny, whereas the reciprocal cross senescent female × non-senescent male (exceptional) yielded senescent progeny. It was inferred that the senescence-determining factor is inside the mitochondria.

14.2.2 Heterokaryon Test

In the heterokaryon test, a heterokaryon is made between a senescent strain and a related nonsenescent strain by mixing and fusion of their germinating conidia or hyphal cells.

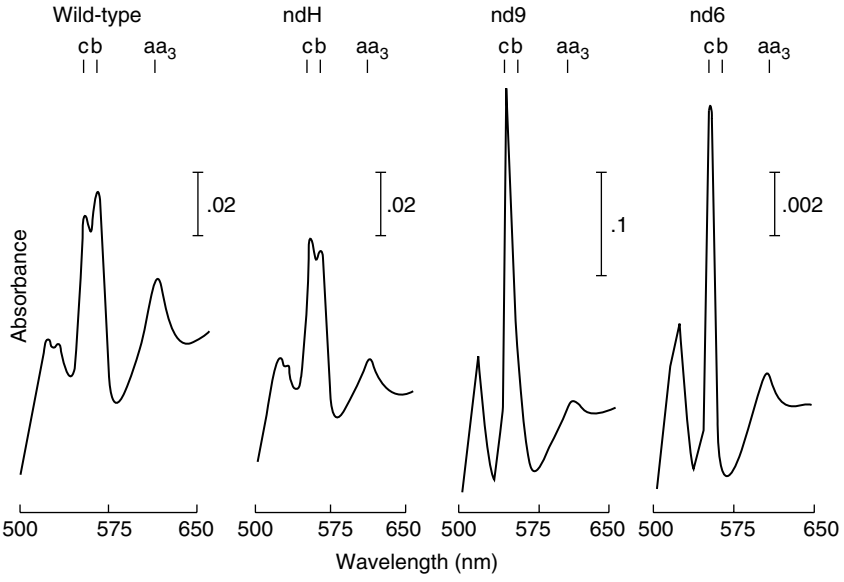

Figure 14.2 Cytochrome content in wild type and senescent nd strains of *Neurospora crassa*. The cytochromes were identified by recording the difference in absorbance between dithionite-reduced and ferricyanide-oxidized mitochondria at wavelengths of light ranging from 500 to 650 nm. The absorbance maxima at 550, 560, and 608 nm correspond to cytochrome *c*, *b*, and cytochrome oxidase aa_3, respectively. The senescent strains nd9 and nd6 are deficient in cytochromes aa_3 and *c*. The senescent strain maintained in a heterokaryon (ndH) has spectra similar to wild-type. (From Seidel-Rogol et al. (1989). American Society of Microbiologists. With permission.)

The nuclei and mitochondria in the fused cell multiply and mix. To force heterokaryotic growth, auxotrophic markers, e.g., pantothenic acid or leucine, are incorporated into the fusing strains by prior crossing. Only the mixed cytoplasmic growth, i.e., heterokaryon (nonsenescent *pan* + senescent *leu*) grows on minimal media due to the complementation of nuclei from the original strains. The heterokaryotic nonsenescent mycelium can be propagated indefinitely. The genetically marked senescing and nonsenescing nuclear types from the heterokayon can be separated by plating conidia formed by heterokaryotic mycelium on appropriate media. For example, if the *pan* cultures senesce on pantothenate-supplemented medium, the senescent determinant is in the cytoplasm (mitochondria).

14.3 SENESCENCE IN *PODOSPORA ANSERINA*

14.3.1 Deletion and Rearrangements in Mitochondrial DNA

Genetic tests in *Podospora anserina* localized the senescence determinant in mitochondria. A restriction enzyme analysis by agarose-gel electrophoresis revealed that mitochondrial DNA (Figure 14.3) from the senescing cultures contained unique circular DNA molecules comprising head-to-tail of monomer 2.05 μM, dimer, trimer, tetramer or pentamer sizes which were termed the *senDNA* (Jamet-Vierny et al., 1980; Wright and Cummings, 1983).

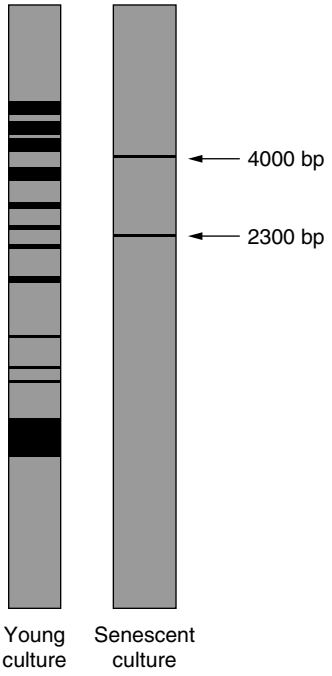

Figure 14.3 Diagram of electrophoresis patterns of mitochondrial DNA from young and senescent cultures of *Podospora anserina*. Arrows point to unique 4000 and 2000 bp fragments in senescent strain after *Hae*III digestion. Based on Jamet-Vierny et al. (1980).

senDNA hybridized to restriction fragments of the mitochondrial DNA but not of nuclear DNA, revealing that it is homologous to mitochondrial DNA (Figure 14.4). Several senDNAs, named α, β, γ, and so on, may be produced by the deletion and amplification of separate regions of mitochondrial DNA, of which senDNAα is produced regularly. senDNAα results from the site-specific deletion and amplification of the first intron of the mitochondrial *COX1* gene which encodes subunit I of the respiratory enzyme, cytochrome *c* oxidase. Wright and Cummings (1983) reported that senDNAα probe hybridized to nuclear DNA from the senescing mycelium and hypothesized that a mitochondrial genetic element is transposed to the nucleus and is integrated into nuclear DNA. However, this observation has not been confirmed—rather, it is likely that the result obtained was due to contamination of nuclear DNA by mitochondrial DNA.

Comparison of nucleotide sequences of mitochondrial DNA fragments revealed intron at new locations in the mitochondrial genome (Sellem et al., 1993). Somehow the defective mitochondria, lacking a portion of mitochondrial genome, increase in number and dominate the cytoplasm during hyphal growth (Figure 14.5)—a condition known as *suppressivity* that results in the loss of ATP production, slowing down of the growth rate and finally in the total cessation of growth. The β and the γ senDNAs are highly variable in size and contain, respectively, an intergenic region downstream of *cox1* gene near rRNA. Their numbers also increase during the progression of senescence but since senDNAα

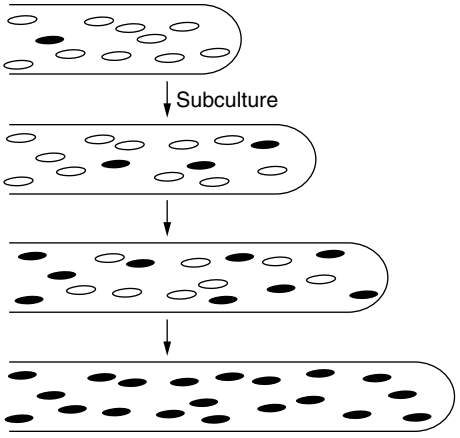

Figure 14.4 A diagram of suppressivity. Defective (closed circle) and functional (open circle) mitochondria.

appears regularly during senescence, it is believed to be the primary determinant of senescence. However, this is controversial because senDNAα was found in nonsenescent mutant *incoloris vivax* (Tudzynski et al., 1982). The uncertainty regarding the role of senDNA is also due to an observation that when senescent mycelium was placed over juvenile mycelium, the senDNAα molecules were transmitted to the recipient mycelium but senescence did not occur (Jamet-Vierny et al., 1999). Moreover, senescence occurred in certain nuclear-gene mutants in which senDNAα amplification was impaired (Dujon

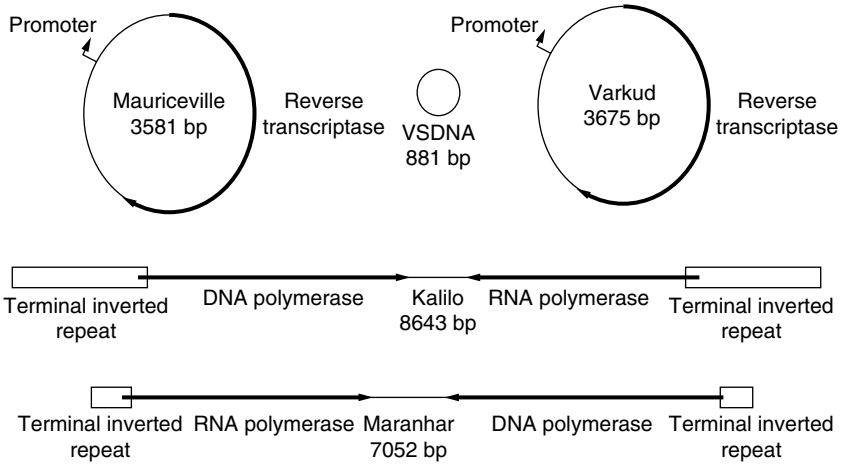

Figure 14.5 pMAU, pVAR, pKAL and pMAR. Heavy lines are open reading frames, with direction of transcription. From Griffiths (1995).

and Belcour, 1989). Because of the inconsequential role of senDNA in senescence, another senDNA—senDNAβ—is suspected to be involved in senescence. Nevertheless, a point of interest is how specific regions of mitochondrial DNA are deleted. PCR analysis of junction sequences of senDNAβ monomers, recovered from several senescent cultures, indicate that the break points are bound by repeats of up to 27 base pairs. It has been hypothesized that deleted mitochondrial DNA molecules arise from unequal intramolecular cross-overs between short repeats that occur in the mitochondrial DNA (Jamet-Vierny et al., 1997a or b).

14.3.2 Nuclear Gene Control of Mitochondrial DNA Deletions

Although senescence in *P. anserina* showed extranuclear (maternal) inheritance, the time of senescence is altered in certain nuclear gene mutants, suggesting that nuclear genes control mitochondrial DNA rearrangements. For example, the double mutant *incoloris vivax* (*i viv*) remained alive for at least four years in contrast to the wild type that died in less than 21 days (Tudzynski and Esser, 1979). The mutant *grisea* also had an extended life span (Borghouts et al., 1997), whereas the mutant *AS1-4* died prematurely in five to six days (Belcour et al., 1991). Since the amplification of senDNAα in the mutant *grisea* was minimal, this suggests that the wild-type GRISEA gene controls the amplification of senDNAα. These observations predict that several nuclear genes control the synthesis of factors which are imported into mitochondria and function in the stabilization of the mitochondrial genome, a point that will be addressed later.

14.4 PLASMID-BASED SENESCENCE IN *NEUROSPORA*

14.4.1 Mitochondrial Plasmids

Plasmids are small molecular weight, autonomously replicating extrachromosomal DNA molecules first discovered in bacteria. In fungi, plasmids are found in the mitochondria where they were discovered in a screen of natural populations of *Neurospora* for structural variants of the mitochondrial chromosome. The presence of plasmids is manifested by a brightly staining band on gels after electrophoresis of restriction enzyme digests of mitochondrial DNA preparations stained with ethidium bromide (Collins et al., 1981). Plasmids (Figure 14.6) are implicated in senescence because they are co-inherited maternally with the senescence character and are integrated into mitochondrial DNA in senescing mycelia (Griffiths, 1995). However, the mere presence of plasmid does not identify a strain as senescence-prone because strains can harbor harmless plasmids.

A survey of *Neurospora* species by the Southern hybridization method using plasmid probes demonstrated that both senescent and nonsenescent strains have two types of plasmids: linear and circular (Griffiths, 1995). The first plasmid discovered was named Mauriceville plasmid after the place from where the host strain was collected. It is a closed-circular DNA molecule (a concatamer of up to six repeats of a monomer of about 3.6 kb). In genetic terminology for plasmid, the letter "p" is added before the first three letters of the name of plasmid in capital letters. In Southern hybridization, the Mauriceville plasmid (pMAU) hybridized neither to mitochondrial DNA nor to nuclear DNA, refuting its origin from either of these DNAs.

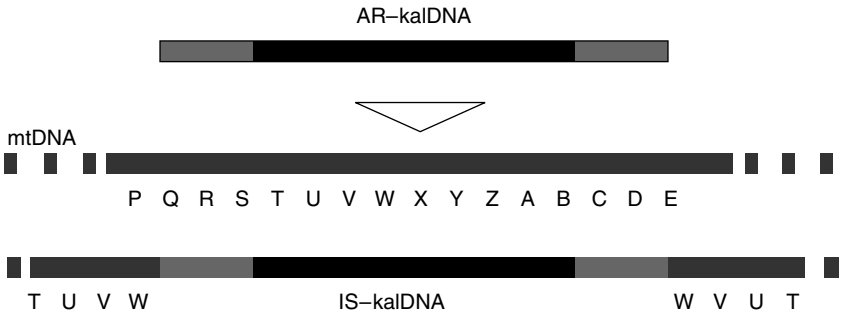

Figure 14.6 Integration of kalilo plasmid into mitochondrial genome. The letters for regions of mtDNA are arbitrary labeled by letters. AR = autonomously replicating plasmid; IS = insertion sequence of plasmid. mtDNA is linearized for purpose of illustration. Adapted from Griffiths (1995).

14.4.1.1 Linear Plasmids

The majority of *N. intermedia* strains collected from Kauai are senescent and were named "kalilo," Hawaiian for "dying." Some wild strains of *N. crassa* collected from Aarey near Mumbai, India, died in about 20 subcultures and these strains were named "maranhar," which in the Hindi language means "prone-to-death" (Court et al., 1991). Kalilo and maranhar strains contain 9- and 7- kb plasmids, respectively. The structural features of the plasmids are deduced from electron microscopy, gel electrophoresis, sensitivity to 5 and 3 exonuclease and sequencing. Both pKAL and pMAR are linear plasmids having terminal regions with inverted repeats of base sequences. A protein is bound to the 5 termini inverted repeats as indicated by resistance to 5 exonuclease digestion (Griffiths, 1995). There are two open reading frames whose amino acid sequences suggest that they encode DNA and RNA polymerases. pKAL and pMAR do not have sequence homology. Based on detection by Southern hybridization, KALILO or homologous plasmids are found in *Neurosporas* collected from different regions of world.

Radiolabeled DNA sequences of pKAL and pMAR hybridized only to mitochondrial DNA from senescing cultures, showing that plasmids inserted into mitochondrial genome —consistent with the maternal transmission of the plasmids DNAs (Myers et al., 1989; Court et al., 1993). The full-length plasmid, flanked by inverted terminal repeats, inserts at a single site into mitochondrial DNA. The plasmids therefore exist in mitochondria in two forms: an autonomously replicating (AR) form and as an insertion sequence (IS), which for the kalilo are denoted mtAR-kalDNA and mtIS-kalDNA, respectively. Plasmids could induce senescence by inserting into mtDNA and inactivating an indispensable gene (Figure 14.7).

14.4.1.2 Circular Plasmids

The Mauriceville strain of *N. crassa* and the Varkud strain of *N. intermedia* have circular mitochondrial plasmids, normally benign but they can mutate into variant forms and become "killer" plasmids. During continuous growth in race tubes or during sequential conidial transfers in slants, these strains showed either "stop-start" growth or senescence.

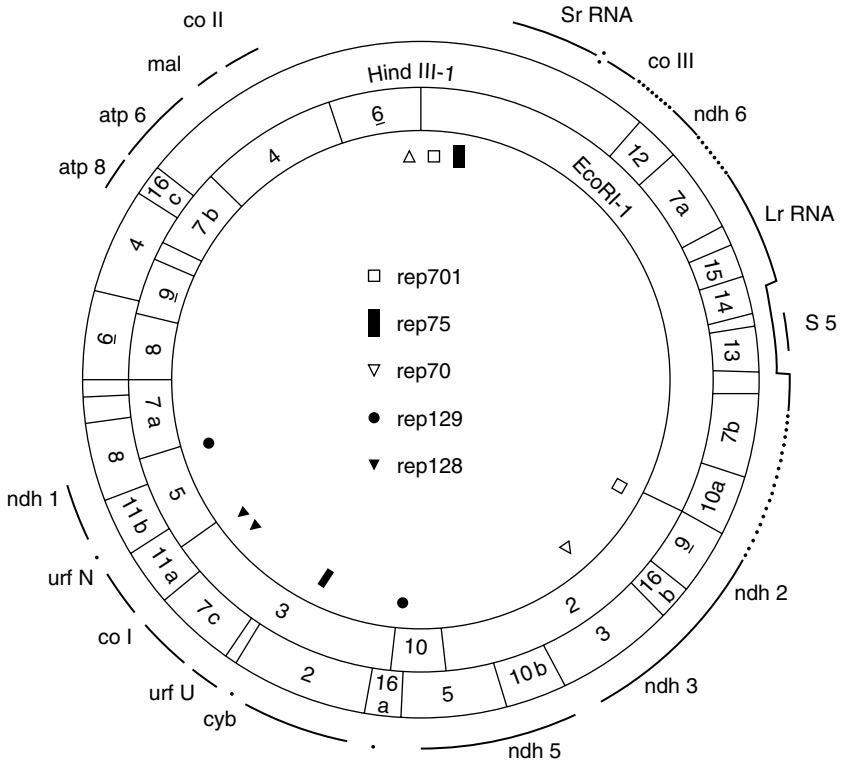

Figure 14.7 Map of wild type *Neurospora crassa* mitochondrial DNA. The outer circle shows the fragments produced by *Hind*III restriction enzyme. The inner circle shows the fragments produced by EcoRI restriction enzyme.

The variant strains had deficiencies of cytochromes aa_3 and *b* (Akins et al., 1986). Densitometry of ethidium bromide stained gel following electrophoresis of total mtDNA shows plasmid accumulation relative to the mitochondrial DNA, i.e., they had become suppressive. DNA sequencing revealed the insertion of a mitochondrial tRNA sequence and short deletions in the plasmid DNA. As both plasmids encode reverse transcriptase, it is hypothesized that during replication, the variant forms of plasmids arise from the benign forms via an RNA intermediate and reverse transcription of full length plasmid transcript that integrates into mitochondrial DNA by homologous recombination (Akins et al., 1989).

14.4.1.3 Spread of Plasmids

Yang and Griffiths (1993) made a global survey of *Neurospora* species by the Southern hybridization method using a plasmid probe and demonstrated that both senescent and nonsenescent strains have mitochondrial plasmids. Although few fungi have been examined for mitochondrial plasmids, it is likely that plasmids are common in fungi. Related plasmids have been discovered in strains collected globally. This raises the interesting question as

to how plasmids spread. One possibility is by hyphal contact of plasmid-harboring and plasmid-lacking strains by transient fusion and mixing of the mitochondria. To test this, a senescent kalilo strain was constructed having the nuclear marker genes *nic-1* and *al-2* (Griffiths et al., 1990). This was mixed with a nonsenescent strain having the marker genes *ad-3B* and *cyh-1*. This heterokaryon was senescent, demonstrating that hyphal fusion could allow the plasmid to spread in nature (horizontal transmission). In some cases, vertical transmission of plasmids has been demonstrated following limited sexual recombination (Bok and Griffiths, 1999). However, the screening of populations over a period of time did not show a change in plasmid frequency. Further investigations are required to determine how the spread of plasmids in nature is checked (Debets et al., 1995).

14.5 NUCLEAR GENE MUTANTS

14.5.1 *natural death*

In *N. crassa*, a single nuclear gene mutant obtained by ultraviolet irradiation was called *natural death* (*nd*). The mutant *nd* dies in 2–4 sequential transfers; therefore, to preserve *nd* cultures for studies, its nuclei are marked and preserved in a heterokaryon in association with the wild type. For example, the *nd* strain is crossed to an albino strain and the white (albino) recombinant progeny (*nd al*) is selected that shows the death phenotype in subcultures. The mutant *nd al* can, however, be indefinitely maintained in combination with nuclei having the wild type (nd^+) allele and when desired recovered by plating conidia formed by the heterokaryon and selecting the white *nd* colonies carrying the *al* marker allele. After extraction, the *nd* homokaryon died again in 2–4 subcultures but this provides the time for sexual crosses and mycelium to be grown for DNA analysis. This technique of preserving potentially lethal mutations possible with *N. crassa* make it very suitable for investigation of the phenomenon of aging and death.

The sequence of 65.5 kb circular mitochondrial DNA molecule of *N. crassa* is known and an EcoRI restriction map is shown in Figure 14.8. On the basis of their migration in agarose gels, 11EcoRI bands can be distinguished. A comparison of the restriction digest profiles of mitochondrial DNA from *nd* and nd^+ mycelia showed unique fragments in the *nd* homokaryons recovered from the heterokaryon that are not present either in the wild nd^+ or in the [*nd* + nd^+] heterokaryons (Seidel-Rogol et al., 1989; Bertrand et al., 1993). The unique EcoRI restriction fragments (Figure 14.9) from mitochondrial DNA were cloned and sequenced. Comparison of its sequence with wild mtDNA reveals that *nd* suffers short deletions and its nucleotide sequences are rearranged. The deleted segments are those that have a palindrome sequence, suggesting that this sequence gets recognized and is removed by specific endonucleases and the separated fragments juxtaposed. It was hypothesized that unequal crossing-over between repeat sequences result in deletions followed by recombination of distant nucleotide sequences (Figure 14.10).

14.5.2 *senescent*

Another nuclear gene mutant *senescent*, derived from a phenotypically normal heterokaryotic wild isolate by extracting nuclei in the form of uninucleate microconidia (Navaraj et al., 2000) and growing them into homokaryotic cultures (Figure 14.11), exhibited the "death"

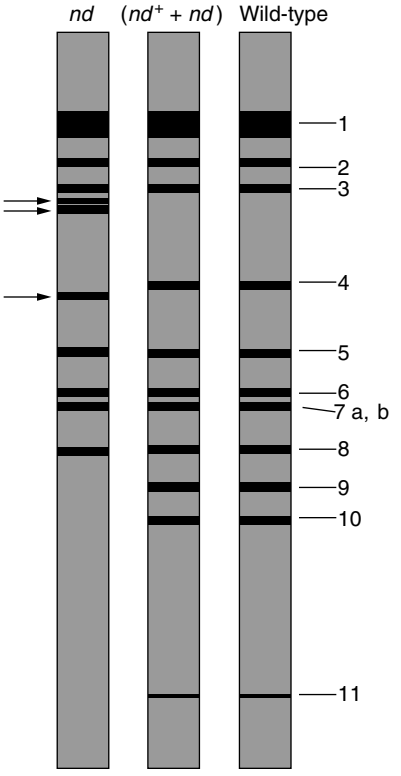

Figure 14.8 Diagram of restriction fragment patterns from mitochondrial DNA of wild (nd^+), *nd* mutant and a [nd^+ + *nd*] heterokaryon. Adapted from Bertrand et al. (1993).

phenotype upon subculturing (Figure 14.12). In crosses of *senescent* × wild type, the mutant and wild type segregated in a 1:1 ratio (Figure 14.13), demonstrating that senescent is a single nuclear-gene mutant. Comparison of restriction fragments of mitochondrial DNAs from sen^+ showed that senescing mycelia suffered deletions and gross sequence rearrangements.

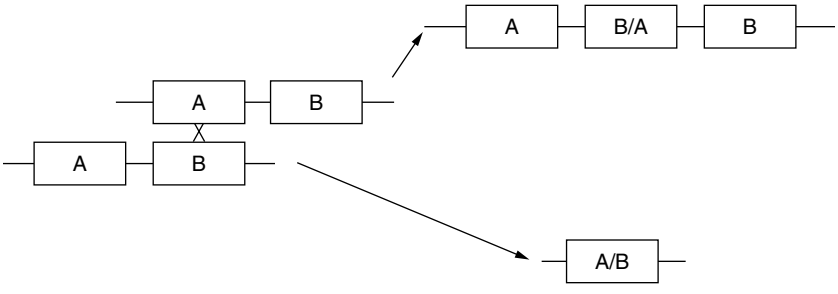

Figure 14.9 Diagram of unequal crossing over between repeat sequences. Based on Bertrand et al. (1993).

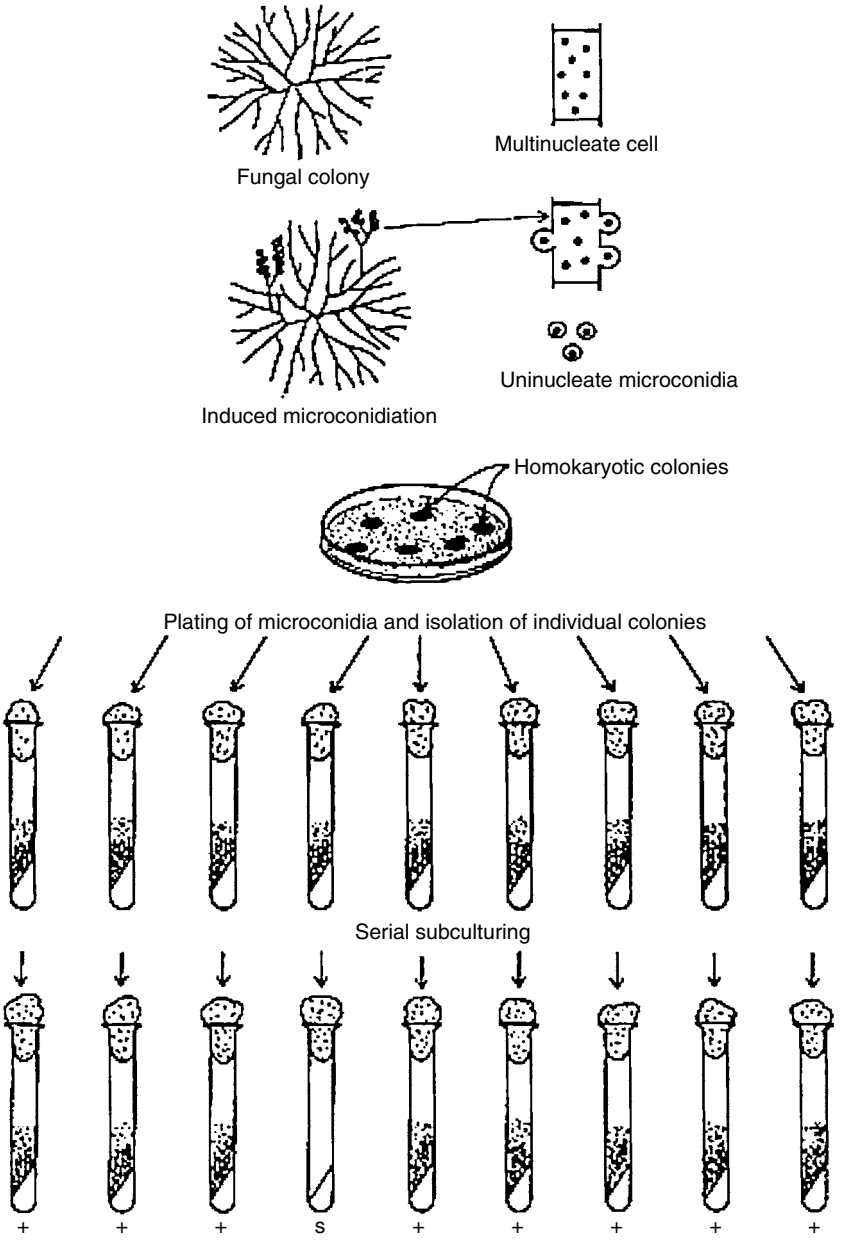

Figure 14.10 Derivation of senescent mutant from a heterokaryotic wild isolate of *Neurospora* using microconidia.

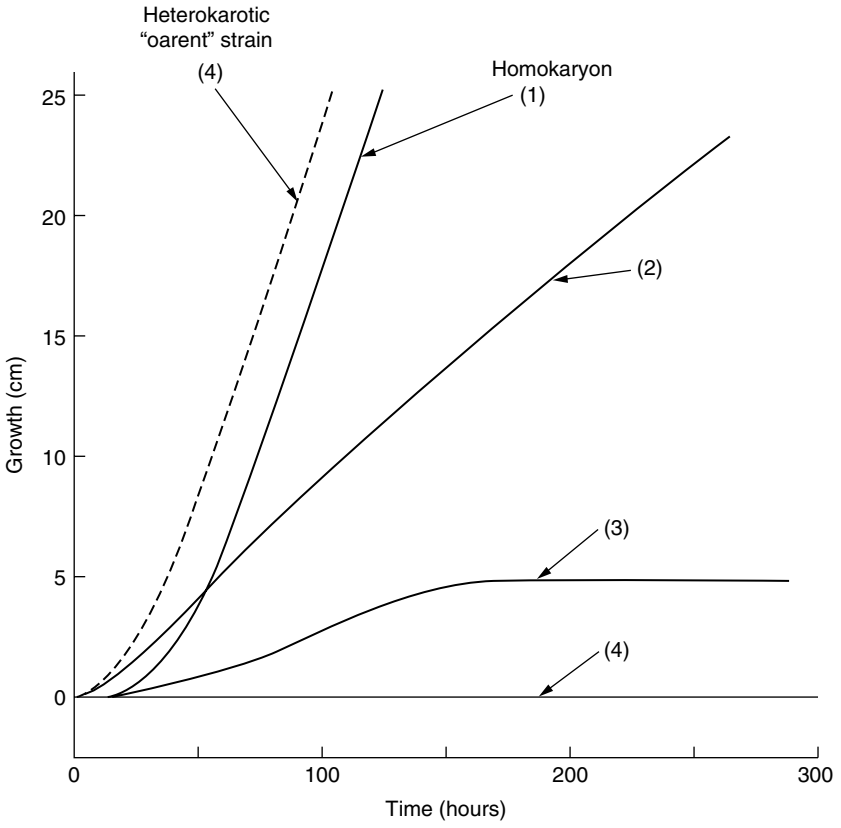

Figure 14.11 Growth rate of senescent mutant. The subcultures numbers (in parenthesis) were made in race tubes.

14.6 AEROBIC RESPIRATION, AGING AND SENESCENCE

Aging and ultimately death occur in all aerobic organisms, though most fungi are seemingly an exception. One widely accepted theory attributes aging and death to cellular damage caused by free radicals generated by normal metabolism. A *free radical* is a molecular species that contains an unpaired electron. Free radicals are highly reactive and destructive because of their tendency to pair up by donating or take up an electron. Free radicals are generated when electrons are incorrectly passed between different molecules in the electron transport chain in mitochondria. When radicals damage enough molecules in the cells, the organisms die (http://www.accessexcellence.org/LC/ST/bgfreerad.html). This is known as the free radical theory of aging.

Senescence is correlated with a switch from cyanide-sensitive, cytochromes-mediated to cyanide-insensitive alternative respiratory pathway—the electron flow at the level of ubiquinone (coenzyme Q) is bypassed and the enzyme alternative oxidase transfers the electron directly to molecular oxygen,

$$O_2 + e^- \; O_2^-,$$

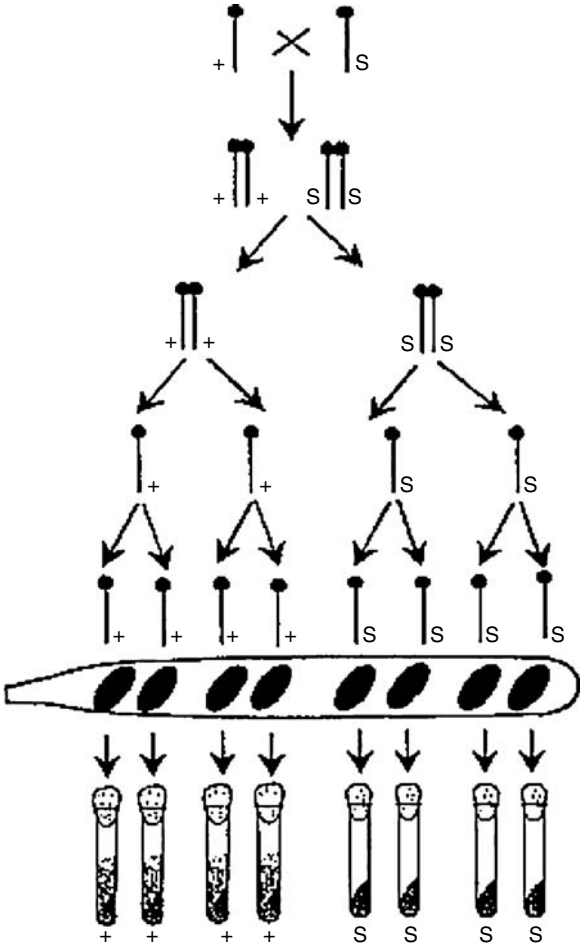

Figure 14.12 Inheritance of a single nuclear gene senescent mutant of *Neurospora*. A *senescent* mutant (*S*) was crossed to a wild strain of opposite mating type.

giving rise to a superoxide anion. A superoxide anion is highly destructive and leads to the formation of hydrogen peroxide, H_2O_2, and hydroxyl radicals, OH^-, collectively called *reactive oxygen species* (ROS). ROS oxidize and damage nucleic acids, lipid and protein molecules and the damage to cells and tissues results in aging and ultimately to death. Since alternative oxidase (Figure 14.14) is located upstream of complex III, the production of ATP is restricted to complex I and consequently the production of ATP is lowered. To determine which of the aforementioned biochemical modifications is the primary cause of senescence, Dufour et al. (2000) examined the effects of specific inactivation of cytochrome oxidase on life-span, ROS formation, senDNA accumulation—modifications implicated in senescence. Since *P. anserina* has (i) a short life-span, (ii) a cytochrome-mediated aerobic respiratory pathway, (iii) an alternative respiratory pathway linking the oxidation of ubiquinol directly to the reduction of oxygen to water and playing a crucial

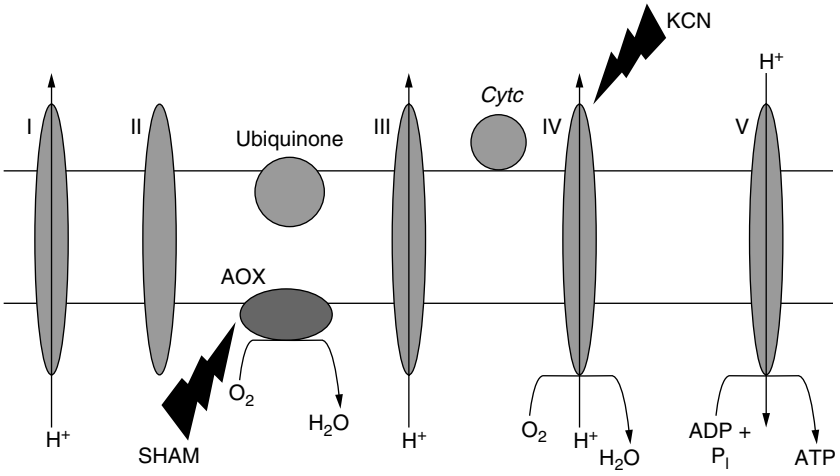

Figure 14.13 Mitochondrial respiratory assemblies that transfer electrons to oxygen. AOX, alternative oxidase, SHAM, salicyl hydroxamate.

role in protecting against the lethal effects of ROS, and (iv) well-developed genetic and molecular techniques, this fungus provided a particularly good system for assessing the role of cytochrome-mediated respiration on the shortening of the life-span. The functional cytochrome gene was replaced by transformation with a plasmid construct that contained cytochrome oxidase gene lacking 169 bp of COX5 gene. Homologous replacement of the functional wild COX5 gene by a defective copy of a synthetic gene prevented the accumulation of senDNAs, decreased the formation of ROS and increased the life span of the fungus. Reintroduction of functional COX5 gene into the deleted strain restored the original phenotype, i.e., the original growth rate, reduced life-span, loss of fertility and increased formation of ROS without the accumulation of senDNAs. The authors hypothesized that aerobic respiration mediated by cytochrome oxidase is the general cause of aging and senescence of eukaryotic organisms. The experimental results favor the free radical theory of aging.

14.7 CONCLUDING REMARKS

The mitochondrial genome is more damage-prone than the nuclear genome, at least in part to damage caused by the generation of a superoxide in the electron transport chain. For hyphal growth to continue, functional mitochondria must continuously replace mitochondria damaged by harmful reactive oxygen species. Since mitochondrial proteins are encoded by mitochondrial and nuclear genomes, the renewal of functional mitochondria requires the integrated action of both the nucleus and the mitochondria. Unexpectedly, the studies of senescence in fungi form a basis for the endosymbiont hypothesis of eukaryotic cells. It is conceivable that during evolution, the transfer of genes encoding mitochondrial proteins protecting the mitochondrial genome were transferred from mitochondria to the nucleus. Cloning and identification of *nd*$^+$ or *sen*$^+$ gene products will have implications in understanding not only the assembly of mitochondria in eukaryotic cells but also in

understanding human mitochondrial diseases due to mutation in nuclear genes. Among the several possibilities are nuclear gene-encoded factors that protect the mitochondrial genome from deletions and recombination events that could occur by default. Alternatively, a nuclear gene mutation that affects the assembly of the multiprotein mitochondrial machinery that translocates proteins from the cytosol into the mitochondria, known as the translocase of outer membrane (TOM) and translocase of inner membrane (TIM), will be at a severe disadvantage in the cell and affect viability. Another possibility is that because of the presence of repeat elements, mitochondrial DNA is inherently prone to intramolecular recombination and deletions and requires "protection" by protein factors. In the course of evolution of eukaryotes, the genes encoding the protective factors could have transferred from the mitochondria to the nucleus. The senescing strains of fungi offer unique opportunities for assessing postulated free radical hypothesis and uncovering supplemental hypotheses of aging and death in higher organisms.

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Appendix

Naming, Defining, and Broadly Classifying Fungi

NAMING OF FUNGI

The scientific name of a fungus follows a binomial system of nomenclature, governed by the International Code of Botanical Nomenclature. It is based on the name of the genus and species with both words italicized. For example, the scientific name of wheat stem rust fungus is *Puccinia graminis* Erikss., in which *Puccinia* refers to the genus, *graminis* is the species, and Erikss. is the abbreviation for Jacob Eriksson, a Swedish mycologist (1848–1931) who first founded the species by publishing the description of the fungus. After first use of full binomial name, the genus name is abbreviated to a single capital letter, as in *P. graminis*. When applicable, an infraspecies category (*forma speciales*) characterized by physiologic criteria (host adaptation) is added after the species name, for example, *P. graminis tritici*. For a name to be accepted, a Latin description must be validly published. Nomenclature is based on the priority of publication. If the vernacular name (e.g., wheat stem rust) is used, the organism should also be identified by the scientific name at least once (e.g., *Puccinia graminis*). Some fungi placed in Fungi Anamorphici (see below) have two names, one based on the asexual stage (anamorph) and another based on sexual stage (teleomorph), for example, *Septoria nodorum* (anamorph) and *Mycosphaerella graminicola* (teleomorph). However, after the connection between the two stages of the same fungus was proven, *Septoria nodorum* is referred to as *M. graminicola*.

DEFINITION OF A FUNGUS

A startling finding from comparisons of sequences of 18S rRNA—a component of the small subunit of ribosomes found in all organisms—was that some species, for example, the long-studied potato late blight fungus *Phytophthora infestans*, are quite distinct from other species of fungi. These species, termed the "pseudo fungi," were given the status of kingdom named Straminipila (or Stramenopila) and distinguished from the "true fungi" placed in the kingdom Eumycota. Other differences too came to light: the vegetative phase in Straminipila is predominantly diploid whereas in Eumycota it is haploid. Moreover, Straminipila reproduce by means of motile biflagellate zoospores, suggesting that they evolved from an alga that lost chloroplasts (Cavalier-Smith, 2001). Their cell wall is composed of cellulose and not of β-(1-3)-glucan or mannan and chitin as in Eumycota.

The Straminipila and Eumycota represent separate domains of life—a situation reminiscent of some forms once included in "Bacteria" but were later separated and grouped in the domain Archaea based on distinct DNA sequences and biochemical features. Because of these advances in our knowledge of the organisms possessing hypha, an issue of debate is how a fungus should be defined, based on the evolutionary origin or on a unique structure? In this book, the fungi are non-photosynthetic, generally multicellular and multinucleate, filamentous eukaryotes encased in a multilayered cell wall. The yeasts, although they are typically unicellular, are included in fungi because their cell wall and reproductive structure (ascus) is very similar to filamentous eukaryotes placed in Phylum Ascomycotina.

CLASSIFICATION

Classification is the placing of an individual in categories. This not only aids in determining whether it is identical or similar to an already known fungus but also in understanding its evolutionary affiliation. Naming and classification go hand in hand. The modern trend is to classify fungi based on genealogy. Some interpretations of relationships based on their evolutionary sequence conflict with the classification schemes that were developed based on morphological characters. A *genus* is a group of closely related species, for example, *Puccinia coronata*, *P. graminis* and *P. sorghi*. A *family* is a group of related genera, an *order* is a group of related families, a *class* is a group of related orders and a *phylum* is a group of several classes descending from a common lineage. However, no stable definition of these categories has yet been adopted for fungi. In particular, there is no uniformity in the name endings of the higher categories. The phylum or subdivision name ends in *mycotina*, the class in *–mycetes*; the order in *–ales*, and the family name in *–aceae*. The classification adopted here is chiefly as given in Burnett (2003). For the black wheat stem rust fungus, its classification and the nomenclature is as given below:

Kingdom/division: Eumycota
Phylum/subdivision: Basidiomycotina
Class: Urediniomycetes
Order: Uredinales
Family: Pucciniaceae
Genus: *Puccinia*
Species: *graminis*
Forma specialis (f.sp): *P. graminis* f.sp *tritici*
Race: *P. graminis* f.sp *tritici* race 56

The infraspecies rank of *forma speciales* designates a variant of the species parasitizing a particular host. A *race* is a category used in pathogenic fungi that is subordinate to *forma* and is similar in form but distinguishable on the basis of pathogenic reaction on varieties of a plant. When first used, the full name of the fungus should be given to avoid confusion. For example, the abbreviated binomial *P. graminis* could also mean *Polymyxa graminis*.

A BROAD CLASSIFICATION OF FUNGI

Kingdom Eumycota

"True" fungi. Predominantly haploid; cell walls with chitin, β-(1,3)-glucans or mannans.

Phylum Chytridiomycotina

Mostly marine forms, single-celled with rhizoids; asexual reproduction by motile zoospores; sexual reproduction unknown.

Phylum Zygomycotina

The hyphae are usually non-septate; sexual reproduction by morphologically undifferentiated gametangia. Gametangial fusion results in reproductive structure called a *zygospore* that presumably germinates by means of a sporangium containing uninucleate spores (Figure A1). Example: *Mucor*, *Rhizopus*.

Phylum Glomeromycotina

Here are placed the mycorrhizal fungi that form symbiotic relationships with plants. Asexual reproduction is by means of chlamydospores. Example: *Glomus*, *Acauleospora*, *Gigaspora*, *Scutellospora*.

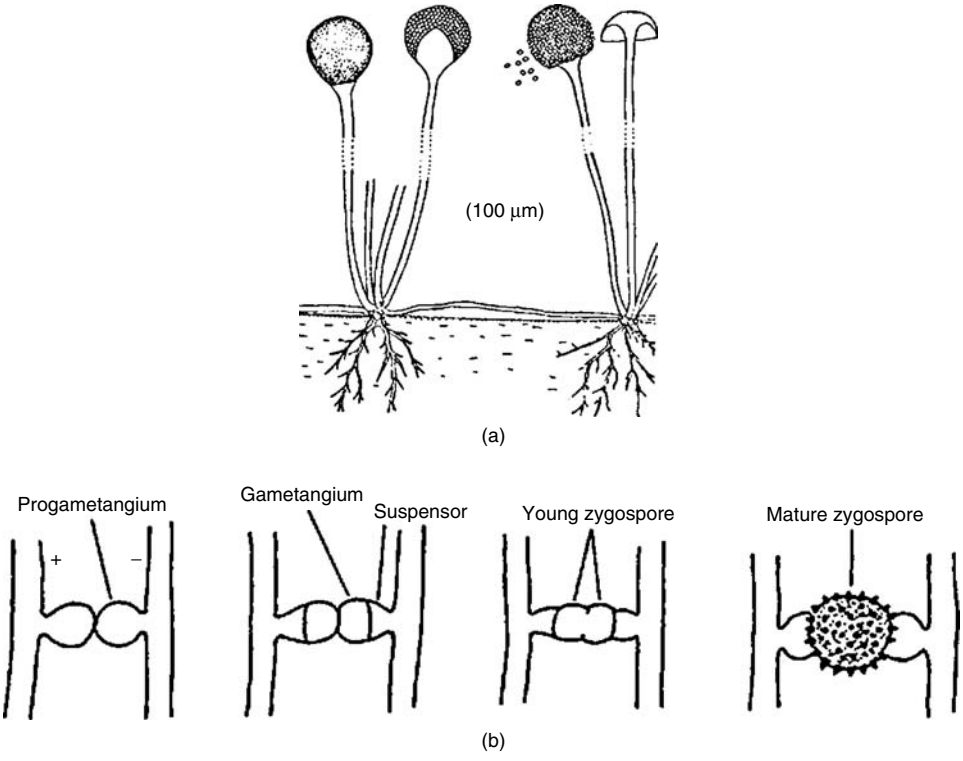

Figure A1 Zygomycotina. (a) *Rhizopus stolonifer*, stolons, rhizoids, young and ripe sporangia. From Von Arx, J.A. (1981), *The Genera of Fungi Sporulating in Pure Culture*. J. Cramer. With permission from Koeltz Scientific Books. (b). Zygospore formation in heterothallic *Mucor hiemalis* by fusion of gametangia. From Ingold, C.T. and Hudson, H.J. (1993). With kind permission of Kluwer Academic Publishers.

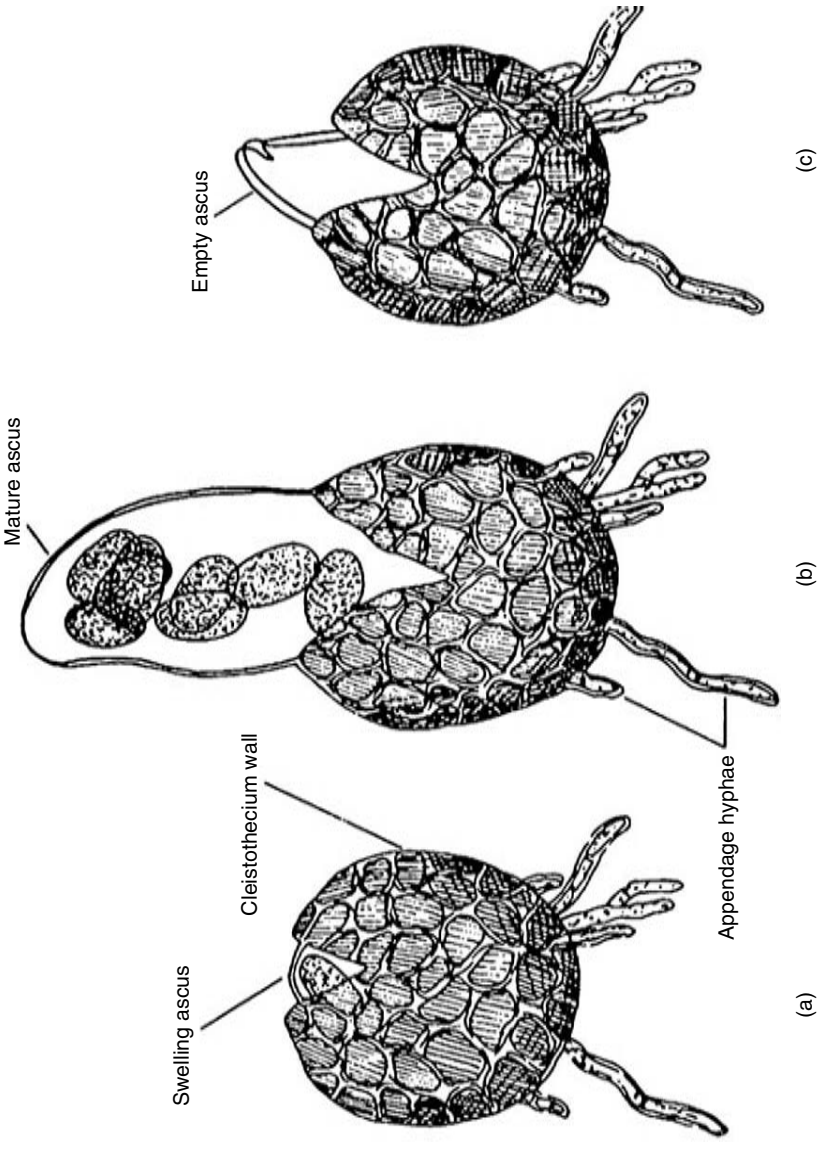

Figure A2 Ascomycotina. (a) A swelling ascus bursting the wall of cleistothecium. (b) Ruptured cleistothecium and ascus. (c) Ascus immediately after discharge of ascospores. (From Ingold, C.T. and Hudson, H.J. (1993). Kluwer Academic Publishers. With permission.)

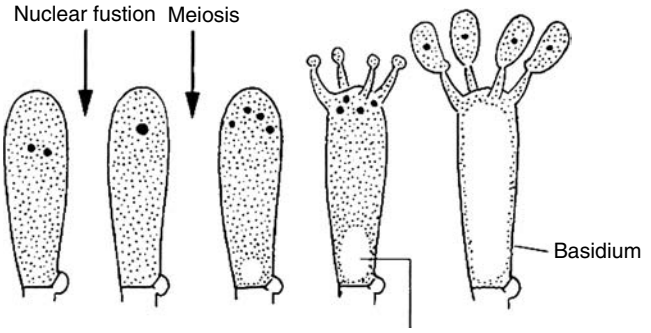

Figure A3 Basidiomycotina. Diagram of basidium development. From Ingold, C.T. and Hudson, H.J. (1993). With kind permission of Kluwer Academic Publishers.

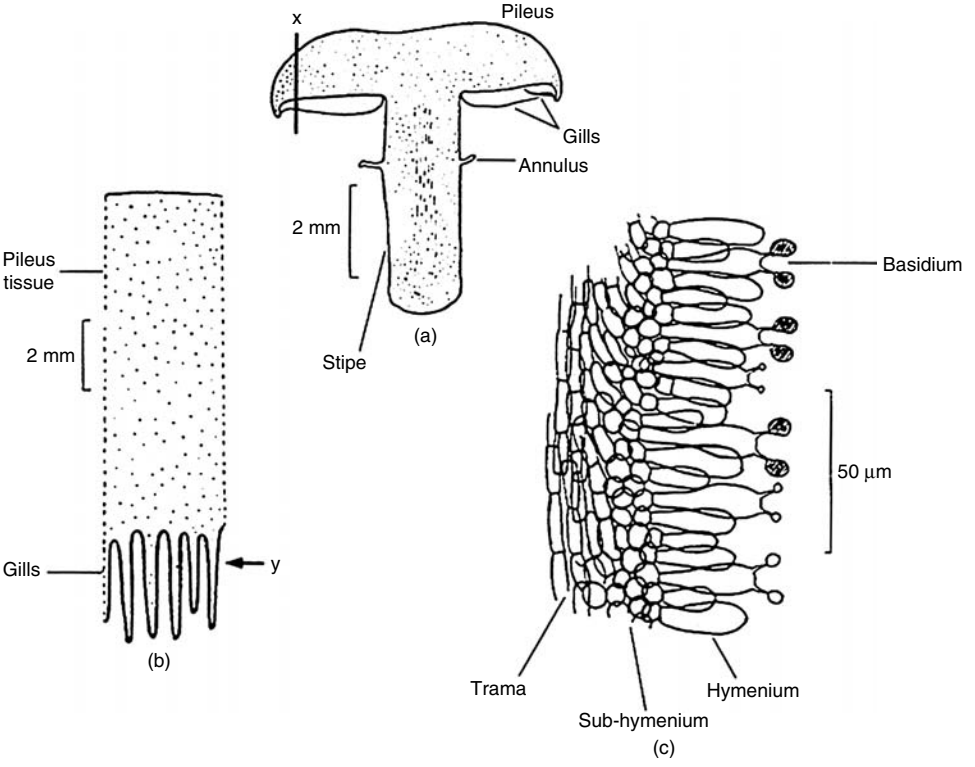

Figure A4 Basidiomycotina. Fruit body (sporophore) with cap (pileus) and lamellae (gills) (a), vertical section of cap (b) showing enlarged view of basidium. From Ingold, C.T. and Hudson, H.J. (1993). With kind permission of Kluwer Academic Publishers.

Phylum Ascomycotina

The hyphae are compartmented, typically branched and septate with septal pores. Asexual reproduction is by means of uninucleate or multinucleate conidia. The sexual spores are produced in a sac called an *ascus* that usually contains four or eight ascospores. Example: *Neurospora*, *Sphaerotheca* (Figure A2).

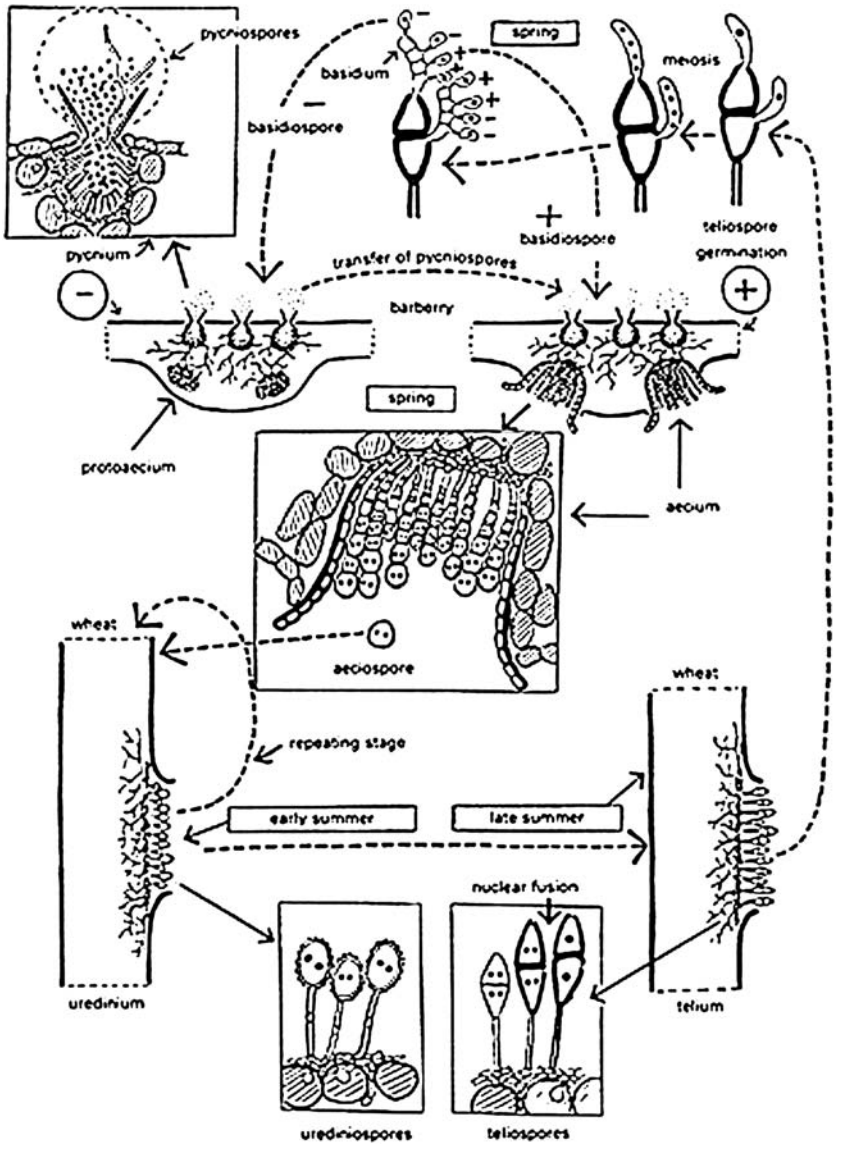

Figure A5 Basidiomycotina. Life cycle of *Puccinia graminis*, a heteroecious, obligate plant pathogen. (From Ingold, C.T. and Hudson, H.J. (1993). *The Biology of Fungi*. Kluwer Academic Publishers. With permission.)

Phylum Basidiomycotina

Comprised of the second biggest group of fungi with some 16,000 species. Hyphae are compartmented with a characteristic clamp connection at septa. The vegetative mycelium is a dikaryon, each cell containing two sexually compatible, haploid nuclei. The fusion of haploid nuclei produces a transient diploid nucleus in a cell called a *teliospore* or *chlamydospore*. The diploid nucleus divides to form four meiotic products called *basidiospores* that, unlike in Ascomycotina, are abjected outside from a tube-like or a club-shaped basidium (Figure A3). Example: *Agaricus bisporus* (Figure A4), *Puccinia graminis* (Figure A5).

Phylum Deuteromycotina (Fungi Anamorphici)

Although a large group of fungi with some 10,000 species, it is a "dust-bin" group into which are placed all those species that have no known sexual stage. The mycelium is

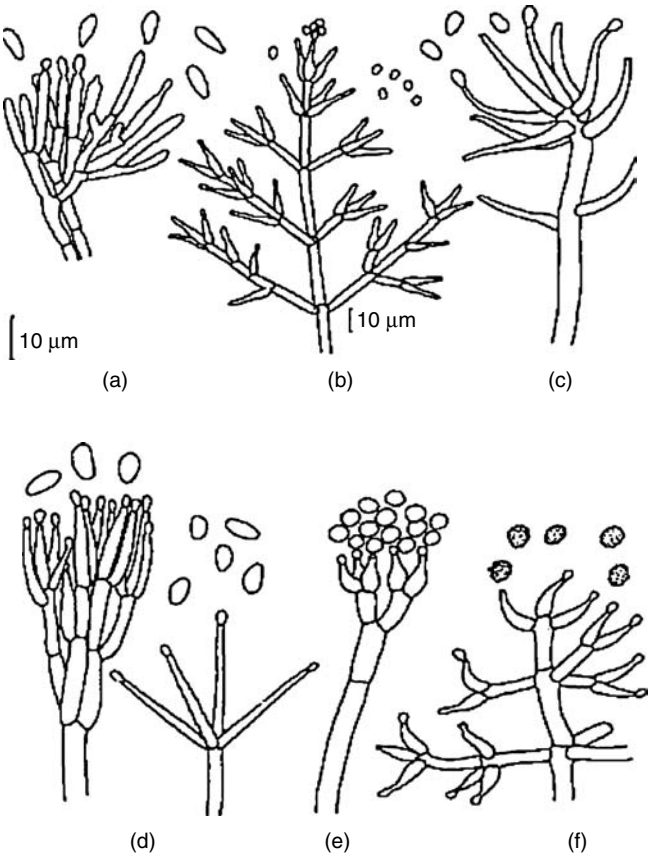

Figure A6 Deuteromycotina. Conidiogenous structures and conidia. (a) *Myrothecium verrucaria*, (b) *Verticillium tenerum*, (c) *Harziella captata*, (d) *Gliocladium roseum*, (e) *Gliocladium virens*, (f) *Trichoderma viride*. (From Von Arx, J.A. (1981), *The Genera of Fungi Sporulating in Pure Culture*. J. Cramer. Koeltz Scientific Books. With permission.)

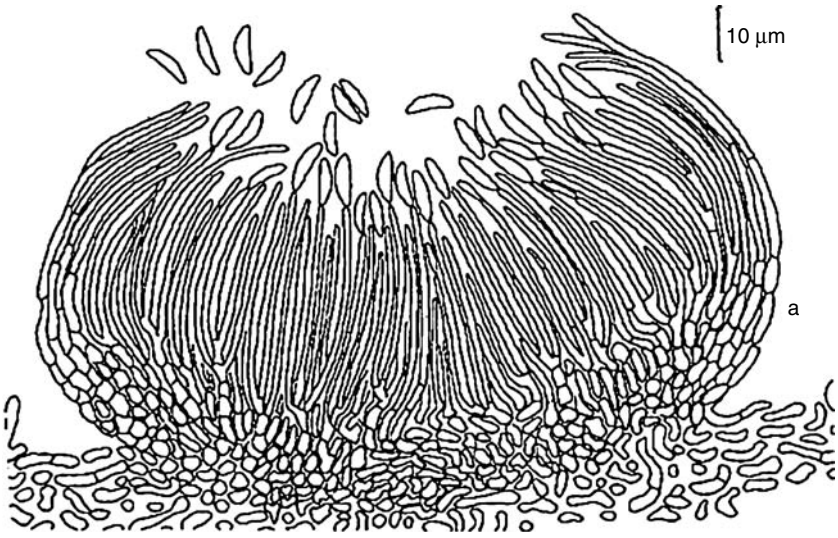

Figure A7 Deuteromycotina. Conidia produced by a cushion of conidiogenous cells (acervulus). (From Von Arx, J.A. (1981), *The Genera of Fungi Sporulating in Pure Culture*. J. Cramer. Koeltz Scientific Books. With permission.)

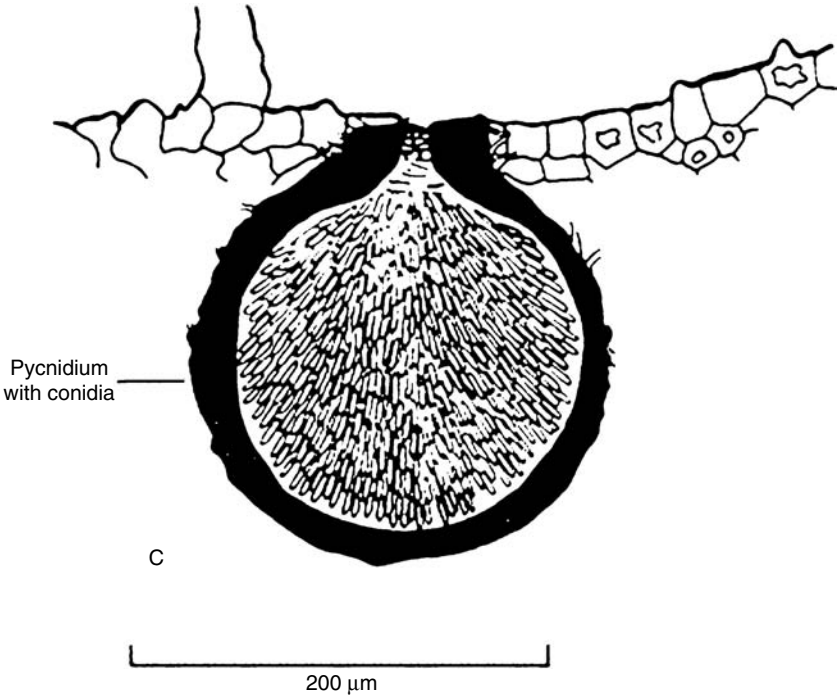

Figure A8 Deuteromycotina. Pycnidium with conidia. (From Ingold, C.T. and Hudson, H.J. (1993). Kluwer Academic Publishers. With permission.)

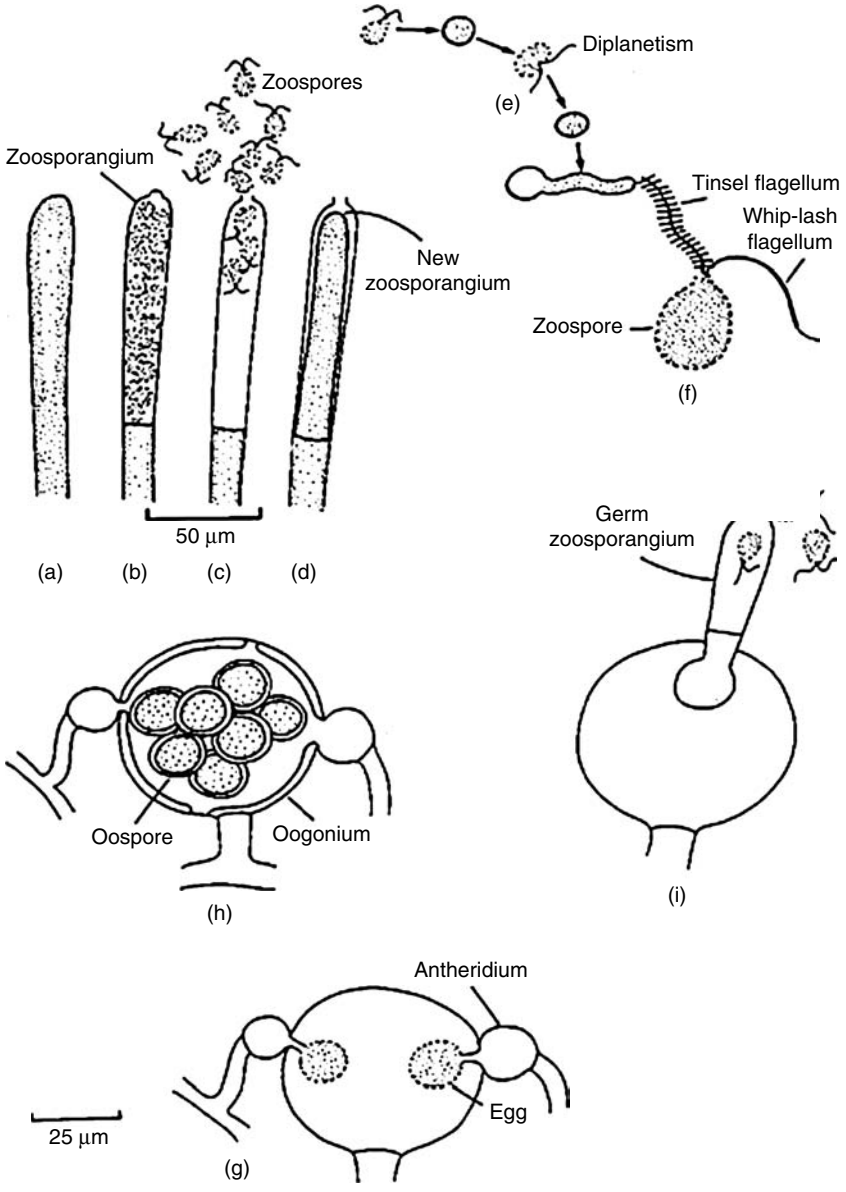

Figure A9 Straminipila. Diagram of (a–c) stages in zoosporangium development of *Saprolegnia*. (d) New sporangium produced by internal proliferation. (e) Encystment and emergence of different form of zoospore (diplanetism). (f) Zoospore with tinsel flagellum directed forwards, and whip-lash flagellum directed backwards. (g) Stages in sexual reproduction. (h) Oogonium with oospore. (From Ingold, C.T. and Hudson, H.J. (1993). Kluwer Academic Publishers. With permission.)

septate, multinucleate, bearing conidia externally on isolated conidiophores (Figure A6), on cushion-like mass of hyphae (Figure A7) or within a flask-like structure (Figure A8).

Some fungi classified in Deuteromycotina have double names, the reason being that the sexual stage was discovered after the binomial given on the anamorphic state became common usage. For example, the sexual (*teleomorphic*) stage of the fungal pathogen of wheat (the glume blotch), *Septoria nodorum*, was recognized 130 years after the fungus had been named on the basis of asexual reproductive structures. This is because the asexual stage (*anamorph*) *Septoria nodorum* occurs on cereals and grasses during spring and summer in England, whereas the sexual stage *Mycosphaerella graminicola* develops in the wheat stubble and litter in the autumn. Until such time that the connection is established, the same fungus is given two names for the conidial and sexual stages and is classified separately. It is a challenging task to connect the anamorph and teleomorph states that are produced at different times or in different situations.

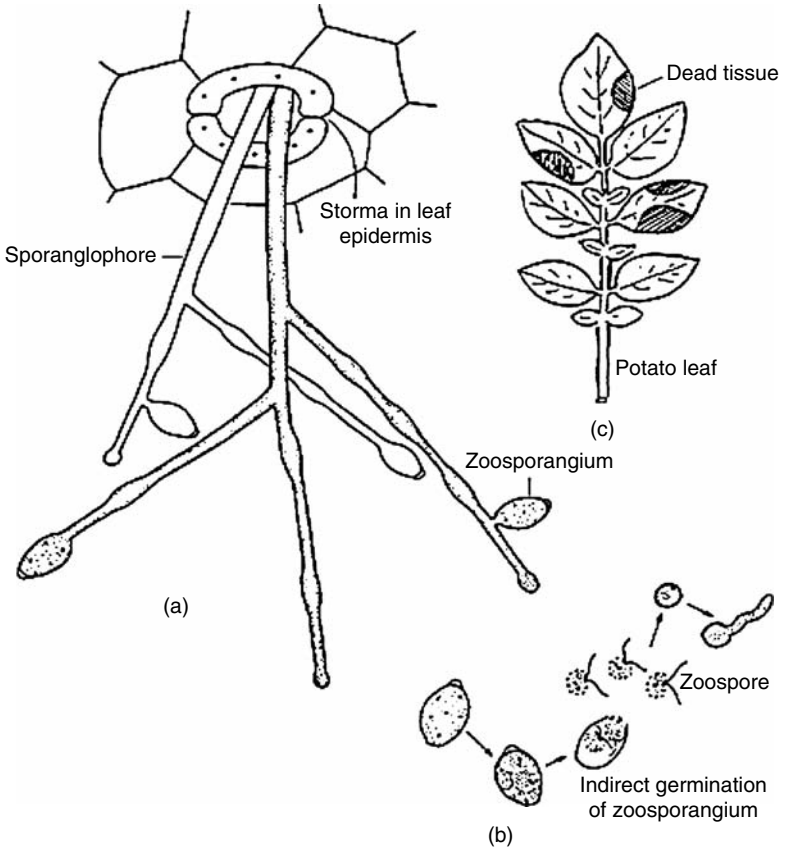

Figure A10 *Phytophthora infestans*, the causal agent of late blight of potato. (a) Surface view of sporangiophore emerging through stoma. (b) Production of flagellated, motile zoospores from sporangium; encystment and germination. (c). An infected potato leaf. (Ingold, C.T. and Hudson, H.J. (1993). With permission Kluwer Academic Publishers.)

KINGDOM STRAMINIPILA (STRAMENOPILA)

Unicellular or hyphal forms; cell walls composed of cellulose-like β-1,4 glucan; lacking septa. Characterized by gametangial meiosis, thus having a diploid vegetative phase predominantly diploid; asexual reproduction by means of motile spores (Figures A9 and A10). Sexual reproduction involves gametes of unequal size. The forms placed here have been called the "pseudo fungi." Includes *Phytophthora infestans*, the causal agent of late blight of potato that provided a great impetus to study of fungi and contributed to development of mycology and plant pathology. However, it is now considered not to be a "true fungus" and is placed in the kingdom Straminipila.

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